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Facilitation of ischaemia-induced ventricular fibrillation by catecholamines is mediated by β 1 and β 2 agonism in the rat heart in vitro

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Facilitation of ischaemia-induced ventricular fibrillation by catecholamines is mediated by β_1 and β_2 agonism in the rat heart *in vitro*

Short title: Facilitation of ischaemia-induced VF

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C D E Wilder and M J Curtis designed the research study

C D E Wilder analysed the data

C D E Wilder and M J Curtis wrote the paper

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Abstract

Background & Purpose

Antiarrhythmic β -blockers are used in patients at risk of myocardial ischaemia, but the survival benefit and mechanisms are unclear. We hypothesised that β -blockers do not prevent ventricular fibrillation (VF), but instead inhibit the ability of catecholamines to facilitate ischaemia-induced VF, limiting the scope of their usefulness.

Experimental Approach

ECGs were analysed from ischaemic Langendorff-perfused rat hearts perfused with adrenoceptor antagonists and/or exogenous catecholamines (313 nM noradrenaline+75 nM adrenaline; CATs) in a blinded and randomised study. Ischaemic zone (IZ) size was deliberately made small or large.

Key Results

In rat hearts with large IZs, ischaemia-induced VF incidence was high in controls. Atenolol, butoxamine and trimazosin had no effect on VF at concentrations with β_1 , β_2 or α_1 adrenoceptor specificity and selectivity, respectively (shown in separate rat aortae myography experiments). In hearts with small IZs and a low baseline incidence of ischaemia-induced VF, CATs, delivered to the uninvolved zone (UZ), increased ischaemia-induced VF incidence. This effect was not mimicked by atrial pacing, and hence not due to sinus tachycardia. However, the CATs-facilitated increase in ischaemia-induced VF was inhibited by atenolol and butoxamine (but not trimazosin), indicative of β_1 and β_2 but not α_1 adrenoceptor involvement (confirmed by immunoblot analysis of downstream phosphoproteins). Furthermore, CATs did not facilitate VF in low-flow globally ischaemic hearts, which have no UZ.

Conclusions and Implications

Catecholamines facilitated ischaemia-induced VF when risk was low, acting via β_1 and β_2 adrenoceptors located in the UZ. There was no scope for facilitation when VF risk was high (large IZ), which may explain why β -blockers have equivocal effectiveness in humans.

Keywords:

Adrenaline; adrenergic; arrhythmia; myocardial ischaemia; noradrenaline; ventricular fibrillation

Abbreviations

ACh, acetylcholine; AD, adrenaline; AP, action potential; AR, adrenoceptor; BG, bigeminy; CATs, catecholamine mixture (313 nM NA + 75 nM AD); CRC, concentration response curve; cTnI, cardiac troponin I; CVD, cardiovascular disease; ECG, electrocardiogram; HR, heart rate; IZ, ischaemic zone; MI, myocardial infarction; NA, noradrenaline; NO, nitric oxide; NOEL, no effect level; PE, phenylephrine; RyR2, ryanodine receptor type 2; SCD, sudden cardiac death; SNS, sympathetic nervous system; TVW, total ventricular weight; UZ, uninvolved zone; VF, ventricular fibrillation; VPB, ventricular premature beat; VT, ventricular tachycardia.

For Peer Review

Introduction

Sudden cardiac death (SCD) contributes >60% of cardiovascular disease (CVD)-related deaths, with a majority of these due to ventricular fibrillation (VF) caused by acute myocardial ischaemia, and the search for targetable mechanisms has proven challenging (Adabag et al., 2010; John et al., 2012).

During the first 30 min of ischaemia (the 'phase 1' period), VF risk is intrinsically proportional to the severity of ischaemia, which is determined primarily by ischaemic zone (IZ) size, set by occlusion location, and by collateral flow (Curtis, 1998; Ridley et al., 1992). Separately, VF risk can be modulated by extrinsic factors such as heart rate (HR) and blood-born factors (Curtis et al., 1993). The relationships between VF incidence and IZ size, and other independent variables, are well-characterized in the rat Langendorff preparation, rendering it a tractable assay system (Curtis, 1998).

Catecholamines have long been regarded as mediators of ischaemia-induced VF with myocardial β_1 agonism the most plausible mechanism; however there are inconsistencies in the published literature with regard to the relationship between the absolute levels of catecholamines accumulating in the ischaemic territory and the incidence of ischaemia-induced ventricular arrhythmias (see discussion for further analysis) (Curtis et al., 1993). Thus, in animals, ischaemia causes catecholamine accumulation in the IZ (Lameris et al., 2000; Schömig et al., 1984) yet the timing does not accord with the timing of VF onset (Curtis, 1998). Adrenoceptor (AR) antagonists inhibit ischaemia-induced VF, yet doses/concentrations tested often lack selectivity for their intended target AR, and effects (e.g. a reduction in ischaemia-induced VF incidence) are better explained by non-specific and even extra-cardiac, off-target effects, as suggested by others (Daugherty et al., 1986; Tölg et al., 1997). Some effects are model-dependent, e.g. off-target β_2 antagonist actions in acutely prepared animals (e.g. rat) elevates circulating blood potassium concentrations resulting in VF suppression, an artefact that does not occur in conscious animals subjected to regional ischaemia when fully recovered from preparative surgery (Botting et al., 1983; Paletta et al., 1989) or indeed in isolated hearts where, due to delivery of Krebs directly to the coronary vasculature and an absence of a circulation, the scope for an alteration in circulating blood potassium is zero. In patients with previous ventricular tachycardia (VT) or VF, and acute myocardial infarction (MI), plasma catecholamines can become elevated (Meredith et al., 1991; Slavikova et al., 2007). β AR antagonists reduce mortality in such patients, but only

moderately (Nademanee et al., 2000; β -Blocker Heart Attack Study Group, 1981), and their specific effects on VF remain unclear, despite decades of use (ISIS-1 Collaborative Group, 1988; The MIAMI Trial Research Group, 1985).

It is important to clarify whether catecholamines are important therapeutic targets for VF suppression or not. It may be possible to reconcile published data if it were the case that catecholamines facilitate phase 1 ischaemia-induced VF rather than *mediate* it. The corollary of this novel hypothesis is that the relevance of catecholamines will depend on the influence of other factors such as IZ size. Here we found that catecholamines acting primarily in the non-ischaemic uninvolved zone (UZ) facilitated ischaemia-induced VF in rat isolated hearts, but only when IZ was small (rendering VF risk low). The mechanism, identified using selective and specific AR antagonists and confirmatory immunoblotting, required dual agonism of β_1 and β_2 ARs. These data explain the likely role of catecholamines in SCD/VF and receptor mechanism, and the limits of achievable protection from AR antagonists.

Methods

Ethical statement

Experiments were approved by the King's College London ethics review board and performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, ARRIVE (Kilkenny et al., 2010), the United Kingdom Home office *Guide on the Operation of the Animals (Scientific Procedures) Act 1986*, and recent guidelines on experimental design and analysis (Curtis et al., 2015). Animal housing and husbandry were exactly as described previously (Andrag & Curtis, 2013), and all test solutions and data analysis were blinded by way of coding. To blind the study, the operator gave a second person a book that listed the groups to be studied. The second person wrote a code by the name of each group (e.g. Brighton, Palace, ManU) and relabelled the stock bottles (identical) with the name of the experimental group. The operator then aliquoted the coded stocks, and the code book was kept by the second person until the experiment was completed and analysed. A total of 620 animals were randomised to groups.

Animals, general experimental methods and experimental strategy

Male Wistar rats (Harlan UK; 220-410g) were anaesthetised by intraperitoneal injection of a lethal dose of sodium pentobarbitone (170 mg kg^{-1}) and co-administered sodium heparin (160 IU kg^{-1}) (Wilder et al., 2016). A surgical level of anaesthesia was determined by the absence of pedal and corneal reflexes, prior to cardiac excision and exsanguination. Hearts were immediately removed and arrested in ice-cold modified Krebs control solution (hereafter called 'Krebs'), containing NaCl 118.5 mM, CaCl₂ 1.4 mM, glucose 11.1 mM, NaHCO₃ 25.0 mM, MgSO₄ 1.2 mM, NaH₂PO₄ 1.2 mM and KCl 3 mM, then mounted on to a metal cannula via the aorta for Langendorff perfusion with gassed and warmed (95% O₂: 5% CO₂; pH 7.4; 37°C) Krebs. All perfusion solutions were filtered (5 µm pore size) before use and delivered at a constant pressure of approximately 80 mmHg (generated by the height of the perfusion columns). A unipolar electrocardiogram (ECG) recording was used for assessment of cardiac rhythm by inserting a wire electrode into the left ventricular wall at the apex of the heart, as described previously (Dhanjal et al., 2013), and ventricular arrhythmias (figure 1) were identified using the Lambeth Conventions II definitions (Curtis et al., 2013).

To obtain coronary occlusion, a silk suture sewn under the left main coronary artery was threaded through a polythene tube, which was tightened and clamped using curved Spencer Wells forceps to induce regional ischaemia, and later loosened to achieve reperfusion (normally 30 min after the onset of ischaemia). To generate an IZ of specific size, a silk suture was placed loosely around the left main coronary artery at one of three predetermined set levels: distal, medial or proximal to the atrial appendage, generating a small, medium or large IZ, respectively (table 1) (Andrag & Curtis, 2013; Ridley et al., 1992).

After reperfusion, the IZ was identified by perfusing hearts with disulphine blue dye (Patent blue VF sodium salt, Sigma Aldrich®, UK), followed by re-occlusion of the left main coronary artery. Hearts were then perfused with dye-free test solution in order to wash out any excess blue dye from the UZ. The IZ and UZ were dissected and weighed, and the IZ was quantified as a percentage of total ventricular weight (TVW). In hearts in which reperfusion was not possible because IZs and UZs were required for immunoblotting (catecholamines + antagonist study), the IZ was judged from the location of the occluder and downstream tissue discolouration, and dissected by eye, with values verified by % reduction in coronary flow following coronary ligation (Curtis, 1998).

The need to generate groups of different IZ sizes is an intrinsic aspect of our experimental strategy and is predicated by the hypothesis that catecholamines facilitate ischaemia-induced VF, and this is feasible only when VF risk is low, meaning that there exists scope for the incidence of VF in a group of hearts to be increased. When IZ is large (table 1) it is well established that in a group of rat isolated hearts, the incidence of ischaemia-induced VF will be high, meaning the scope for facilitation of VF will be too low for facilitation to be pathophysiologically relevant, or indeed detectable by statistical analysis in a typical study of no more than $n=20$ hearts/group (Curtis, 1998). In addition to statistical considerations, evaluating possible facilitation of VF in hearts with smaller IZ sizes makes biological sense since it is known that numerous factors appear to play a role in arrhythmogenesis but that their relevance becomes less as VF risk becomes greater owing to 'optimization' of arrhythmogenic conditions, e.g., as a consequence of the IZ size being large (Curtis, 1998; Curtis et al., 1993). Arrhythmogenesis is complex and there is mediator redundancy, known as 'pathophysiological reserve' whereby numerous factors may be sufficient for arrhythmogenesis; this means that each individual factors will not be necessary for arrhythmogenesis under all conditions (Curtis, 1998; Curtis et al., 1993). This is nuanced, but

the nuance is important, and addressing it distinguishes our objectives from the more simplistic and fallacious concept of catecholamines ‘causing’ ischaemia-induced VF.

Experimental protocols

Throughout each experimental protocol, a range of ancillary variables including HR, coronary flow, PR and QT interval at 90% repolarization (QT₉₀), were recorded every 5 minutes, and the occurrence of ventricular arrhythmias was noted in each 5 minute time interval. The QT₉₀ is recorded in rat studies because the T wave is not distinct and the QRST is a single complex that returns to isoelectric asymptotically, rendering the conventional QT interval (at 100% repolarization) very difficult to measure accurately; use of QT₉₀ is validated and long established (Rees & Curtis, 1993; Ridley & Curtis, 1992). An arrhythmia score was assigned to each heart for ischaemia and (separately) for reperfusion in order to permit parametric statistical analysis. All these assessments were as described previously (Wilder et al., 2016).

Hearts were perfused initially with Krebs for 5 minutes and excluded if predetermined criteria were not met (Wilder et al., 2016). In total, 38 hearts were excluded and these were immediately replaced to maintain group sizes while maintaining the randomization (see below). Baseline recordings were then made, followed by switch to test solution that varied according to study subset. In the initial study to examine the effects of catecholamines on VF in hearts with different predetermined IZ sizes, these were vehicle (Krebs + 50 μ M ascorbate) or catecholamine mixture (CATs: 313 nM noradrenaline [NA] and 75 nM adrenaline [AD] + 50 μ M ascorbate). In subsequent studies to identify adrenoceptor antagonists with no non-specific off target pharmacology (in hearts perfused *without* CATs) these were vehicle (Krebs), 1 or 10 μ M atenolol, butoxamine, prazosin, terazosin or trimazosin. In studies to identify the receptor mechanisms involved in mediating CAT effects on VF, these were vehicle (Krebs + 50 μ M ascorbate), CATs or CATs + 1 μ M atenolol, butoxamine or trimazosin. The experimental details for these groups are shown in (figure 2). The CATs mixture used restores HR in Langendorff perfused hearts to rates seen in conscious rats (Curtis et al., 1985), and the concentration ratio of NA to AD is representative of the ratio encountered in human plasma (Baumgartner et al., 1985; Goldstein et al., 2003), especially under conditions of stress (Coplan et al., 1989; Ratge et al., 1986). We have explained the basis for the CATs mixture more fully, and characterised and validated its use in a previous

study focused on the role of CATs in mediating VF during infarct evolution (which occurs after periods of ischaemia of more sustained duration than necessary to evoke so-called phase 1 arrhythmias, which are the focus of the present study) (Clements-Jewery et al., 2009).

Ten minutes after solution switch/initiation of pacing, the coronary ligature was tightened to induce regional ischaemia, which was maintained for 30 minutes and terminated by reperfusion. Although not the main focus of the present study, this allowed examination of CAT effects (in hearts perfused *with* CATs) on reperfusion-induced VF, with further monitoring of ancillary variables for 10 minutes (figure 2). Of each group in the pacing study, 50% of hearts were reperfused to enable future sub-analysis of cardiac biochemistry between time-matched reperfused and ischaemic tissue, using snap frozen samples (-80°C). Hearts in the catecholamines + antagonist study were not reperfused because IZs and UZs (dissected and frozen in liquid nitrogen) were required for later immunoblot analysis.

Supraventricular pacing

In order to establish or rule out whether CATs facilitate ischaemia-induced VF via effects on HR, a separate group of Krebs' perfused hearts (figure 1) was paced via the right atrium at a rate to match that of the average pre-ischaemia rate caused by CAT perfusion (found to be 405 beats min⁻¹) using a silver wire bipolar electrode, connected to a stimulator (CD-S103, Cudos, UK). The stimulator was linked to a PowerLab™ system (PowerLab 4/35 and Animal Bio Amp, ADInstruments, UK; sampling rate 1 kHz), and hearts were stimulated with square wave pulses at double the threshold pulse width (0.34 ± 0.05 ms duration), and double the threshold voltage (1.75 ± 0.2 V) at 6.75 Hz. Regional ischaemia was induced as described above, to generate small IZs, allowing scope for examining whether sinus tachycardia matching that evoked by CATs was sufficient to increase the incidence of ischaemia-induced VF.

Low flow global ischaemia

With *regional* ischaemia in the rat isolated heart, there is limited scope for delivery of exogenous CATs to the IZ owing to the uniform low level of coronary collaterals that delivers a flow to the IZ that is no more than 5% of flow in the UZ (Curtis, 1998). This means that the aforementioned regional ischaemia studies allow testing only of whether CATs acting

in the perfused UZ facilitate ischaemia-induced VF. Therefore, to test the possibility that, with a more substantial level of collateral flow, CATs may additionally act in the IZ to facilitate ischaemia-induced VF, required a different model. We therefore elaborated a low flow global ischaemia model in which the IZ is continuously perfused to allow CAT delivery. Importantly, any facilitation of ischaemia-induced VF by CATs in a low flow global ischaemia model can occur *only* by actions mediated in the IZ (*there is no UZ*).

Hearts were dissected and cannulated, and an ECG lead positioned as described above. Following 15 minutes of baseline perfusion with Krebs, the perfusate was switched to test solution and coronary flow was reduced to one of three set levels for 60 minutes: severe (0.8 ml min^{-1}), moderate (2 ml min^{-1}), or mild (4 ml min^{-1}) low flow global ischaemia using fixed resistance (clamps) to regulate inflow to the cannula (figure 2). A group of time-matched regionally ischaemic hearts with large IZs was included in the study to act as a positive control for comparative purposes (figure 2). Hearts were perfused with vehicle (Krebs + $50 \mu\text{M}$ ascorbate) or CATs. HR, coronary flow, RR and QT₉₀ interval, ventricular arrhythmias, and heart temperature were recorded at 5 minute intervals throughout the protocol. The duration of ischaemia was extended beyond the 30 min sufficient to allow evaluation of phase 1 arrhythmias in the regionally ischaemic heart in order to provide a characterization of the arrhythmia profile, since this rat Langendorff low flow global ischaemia model is new and uncharacterized. Thus the experiment was terminated after 60 min of ischaemia.

AR antagonist specificity and selectivity and use of tissue bath myography

Selection of AR antagonist concentrations was guided by published IC₅₀ values, but in order to be sure that the chosen agents possessed specificity (effects attributable to an action on the intended AR), and selectivity (effects attributable *only* to an action on the intended AR), a dual approach was taken. Specificity was identified in two ways: (i) from AR effects on well-established CAT responses in perfused hearts (for example, inhibition of CAT-induced sinus tachycardia denoting β_1 antagonism) and (ii) from separate blood vessel bioassay studies. For the latter, concentration response curves (CRCs) were constructed using isolated rat thoracic aortae and tissue bath myography (Myobath II 4-channel tissue bath, WPI, UK). Thoracic aortae were dissected from anaesthetised male Wistar rats (Harlan UK; 350-400g), and immediately immersed in cold Krebs-Ringer solution, containing: NaCl 118 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5 mM, glucose D (+) 11.1 mM, NaHCO_3 25mM, MgSO_4 1.2 mM, KH_2PO_4 1.2 mM, and

KCl 4.8 mM, cleared of perivascular connective and adipose tissue, and cut into 4 x 3 mm length rings using 5 scalpel blades mounted together (equidistance apart). Aortic rings were denuded (endothelium removed) for α_1 AR antagonist experiments in order to preclude β AR agonism-induced NO-mediated vasodilation (Brawley et al., 2000). An adapted 21 gauge needle was inserted into the vessel rings and rolled gently against the dissecting dish 10 to 15 times in order to remove endothelia.

Aortic rings were mounted and secured via a pair of intraluminal steel wires, and submerged in 15 ml tissue baths containing gassed and warmed (95% O₂: 5% CO₂; pH 7.4; 37°C) Krebs-Ringer solution. CRCs were recorded using LabChart v7 software (ADInstruments, UK) and a PowerLab™ system (PowerLab 4/25 and Pod Expander, ADInstruments, UK; sampling rate 100 Hz).

After a warm-up period of 60 minutes, during which time baseline vascular tension was set, vascular function was tested (achievement of a vasoconstriction to 60 mM KCl within 2 SDs from the study mean). Endothelium integrity was then assessed by pre-constricting with 1 μ M phenylephrine (PE), with a reversal of >70% of the PE-induced tone by 10 μ M ACh taken to indicate the presence of intact endothelium (β AR constriction experiments), and a reduction of <20% taken to indicate the absence of endothelium (denuded vessels; α AR relaxation experiments). Each bath was subsequently washed out 3 times with Krebs-Ringer solution, and left for 15 minutes for tension to return to baseline. Vehicle (water), 1 or 10 μ M AR antagonist (atenolol, butoxamine or trimazosin, volume-matched to vehicle) was added to each tissue bath and left to incubate for 30 minutes. In β AR CRC experiments, aortic rings were pre-constricted with 1 μ M PE, and the response left to plateau (approximately 15 minutes). The PE equilibrium constriction was defined as the tension immediately before the start of the agonist CRC, and was normalised to zero. Subsequently, cumulative β AR agonist CRCs were constructed by sequentially increasing the agonist concentration by 0.5 log units (dobutamine [β_1 AR] or salbutamol [β_2 AR]; 30 nM to 1 mM) in each tissue bath. Vessels were exposed to each concentration of agonist for 6 minutes before the next addition. In α AR CRC experiments in which aortic rings were not pre-constricted, α AR agonist CRCs were constructed by sequentially increasing the concentration of PE by 0.5 log units (30 nM to 1 mM). Vessels were exposed to each concentration of PE for 2 minutes (30 nM to 100 μ M) or 5 minutes (300 μ M and 1 mM) before the next addition.

Selectivity was established by a two-step process that tested an AR concentration that possessed specificity for a *lack* of non-selectivity. Here, non-selectivity is defined as the ability to inhibit ischaemia-induced VF in the *absence* of exogenous CATs. The complete strategy is as follows. When a concentration was shown to obtain expected evidence of specificity based on the drug's conventional description (i.e., a β_1 antagonist causing inhibition of CAT-induced sinus tachycardia, or an α_1 antagonist causing inhibition of CAT-induced vascular constriction in myograph studies) we tested this concentration to determine that it had no effect on ischaemia-induced VF in rat hearts perfused without exogenous CATs. To achieve the latter, rat hearts were subjected to coronary ligation by the method described above but with all ligatures located proximally to evoke a large IZ. Any AR antagonist shown to reduce ischaemia-induced VF incidence at the chosen concentration was deemed to lack selectivity at that concentration, since ischaemia-induced VF in hearts perfused without added CATs is CAT-unrelated by definition, and any suppression of such VF cannot be attributed to adrenoceptor antagonism. Several concentrations of AR antagonist were tested in separate hearts to determine the threshold concentration for any such loss of selectivity, defined from the No Effect Levels (NOELs) on VF incidence.

Only AR antagonists at concentrations showing sufficient evidence of specificity and selectivity were chosen for subsequent studies to test for inhibition of facilitation of ischaemia-induced VF by CATs in hearts with smaller IZs, using the technique and methods outlined above.

Immunoblotting

To provide further evidence that the ARs chosen for study possessed the necessary specificity and selectivity, we probed for downstream signal indicative of AR agonism. For reasons explained in Results it was necessary to probe only for β_1 and β_2 signal. Frozen UZs and IZs from the catecholamines + antagonist study ($n = 5/\text{group}$, hearts chosen at random) were ground to a fine powder and homogenised in EDTA-free lysis buffer. Protein concentration in lysates was determined by performing the Bradford protein assay (Bradford, 1976). Following the addition of sample buffer (Ehler et al., 1999), samples were heat-denatured at 95°C for 5 minutes, and subjected to SDS-PAGE (10% acrylamide for cTnI, or 6% acrylamide for RyR2). Proteins were subsequently transferred to PVDF membranes by electrophoretic transfer using a semi-dry (cTnI) or wet transfer (RyR2) method. For

immunoblot analysis of proteins, membranes were blocked in 10% (w/v) non-fat skimmed milk (Marvel, Premier Foods, UK) in TBST for 1 hour at room temperature, except for membranes containing RyR2, which were blocked in 5% (w/v) BSA in TBST for 1 hour. Membranes were incubated with primary antibodies overnight at 4°C, and were subsequently incubated with secondary antibodies for 2 hours at room temperature. Protein bands were detected with ECL Western Blotting Detection Reagents (GE Healthcare, UK). Films were developed in a Fuji X-ray film processor (RGII, Fuji), and protein band density determined by Quantity One 1D analysis software (Bio-Rad, UK) using a GS-800 Calibrated Densitometer (Bio-Rad, UK).

Data and statistical analysis

The following data and statistical analysis comply with published recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Group sizes were selected on the basis of expected control incidence of VF and the feasibility of detecting an effect at $p < 0.05$, using a published design strategy that allows for data sub analysis to ensure that group sizes are not extended to the chosen maximum (n=20/group) if there is no scope for an effect approaching statistical significance (Andrag & Curtis, 2013). Gaussian distributed variables were subjected to t-tests (where appropriate – 2 groups, 1 time point), or ANOVA (1 way or 2 way with repeated measures where applicable), followed by Dunnett's, Tukey's or Sidak's *post hoc* tests (as appropriate) if F was significant, using GraphPad Prism 6 software. Such values were expressed as mean \pm SEM. Western blot densitometry values were normalised to the 'sum of the replicates' (Degasperi et al., 2014) to minimise the effect of sources of variation, before being subjected to statistical analysis. Binomially distributed variables (arrhythmia incidence, classified as 'did' or 'did not' occur) were compared using Fisher's exact test. $P < 0.05$ was defined as statistically significant.

Drugs and materials

(-)-Adrenaline, (RS)-atenolol, dobutamine hydrochloride, prazosin hydrochloride, salbutamol and terazosin were obtained from Abcam Biochemicals®, UK; butoxamine hydrochloride, (-)-noradrenaline, patent blue VF sodium salt and (+)-sodium L-ascorbate were obtained from Sigma-Aldrich, UK; trimazosin was a gift from Pfizer, UK (compound transfer program). All

western blotting solutions and chemicals, and Krebs perfusate salts were obtained from Fisher Scientific, Sigma-Aldrich, UK, and VWR International, UK. Solutions and buffers were made up in distilled water supplied by a PURELAB flex dispenser and PURELAB Option-Q (ELGA LabWater, UK), with a resistivity of 18.2 M Ω .

Primary antibodies used for western blotting were obtained from the following suppliers: rabbit polyclonal anti-cTnI (4002) and rabbit polyclonal anti-p-cTnI Ser23/24 (4004) from Cell Signaling Technology; rabbit polyclonal anti-calsequestrin 2 (Casq2; PA1-913) and mouse monoclonal anti-RyR2 (MA3-925) from Thermo Fisher Scientific; rabbit polyclonal anti-p-RyR2 Ser2808 (A010-30) from Badrilla, and mouse monoclonal anti- α -actinin (A7732) from Sigma-Aldrich. The secondary antibodies used for western blotting were obtained from the following suppliers: goat polyclonal anti-rabbit (7074) from Cell Signaling Technology, and goat polyclonal anti-mouse (P0447) from Dako.

Results

Catecholamines facilitated ischaemia-induced VF when IZ was small

Varying the location of the coronary ligature obtained the desired IZ sizes, and these were not affected by CATs (figure 3A).

Ventricular arrhythmias

CATs increased ischaemia-induced VF incidence but only in hearts with small IZs, in which control VF incidence was low (figure 3F). Other arrhythmias, more prevalent even with small IZs, were not facilitated (figure 3B-E). **During reperfusion, the pattern of facilitation was the same with CATs increasing VF incidence in hearts with small IZs from 12 to 56%.**

Haemodynamic and ECG changes

CATs increased HR (figure 4A-B) and caused PR shortening (figure 4C), as shown previously in perfused rat and mouse hearts (Clements-Jewery et al., 2002; Stables & Curtis, 2009). Coronary ligation had no effect on HR (figure 4A) but did lower coronary flow, the extent of which was dependent on IZ size as expected (Curtis, 1998) (figure 4B). Reperfusion had no effect on HR (figure 4A), but caused reactive hyperaemia in all groups 1 minute after the start of reperfusion (31 minutes; figure 4B).

Coronary ligation transiently shortened QT interval in vehicle controls during the first minute of ischaemia, an effect that had resolved by 5 minutes of ischaemia and was prevented by perfusion with CATs (figure 4D).

Pacing to mimic effects of catecholamines on HR did not facilitate VF

Pacing at 405 beats min⁻¹ maintained HR throughout the experimental protocol (e.g., vs. 259 ± 10 beats min⁻¹ in non-paced hearts 10 min after the onset of ischaemia, P<0.05). However, pacing did not increase the incidence of any type of ischaemia-induced arrhythmia (figure 5A-D). IZ sizes were similar between non-paced (10.5 ± 0.9% TVW) and paced hearts (10.3 ± 0.8% TVW) as intended and expected.

Identification of AR antagonist concentrations lacking off target pharmacology

In hearts with large IZs and no added CATs, the α_1 AR antagonists, prazosin and terazosin, significantly reduced ischaemia-induced VF incidence: prazosin from 67% (vehicle) to 17% (1 μ M) and 8% (10 μ M), and terazosin from 58% (vehicle) to 8% (10 μ M). Additionally, prazosin and terazosin reduced HR and coronary flow, and prazosin also affected ECG intervals (figure 6). All these effects are indicative of AR-*independent* off target actions (Clements-Jewery et al., 2002; Grimm & Flack, 2011; Thandroyen et al., 1983), the nature of which are not relevant to the present study, but which rendered these drugs unsuitable as selective probes for the α_1 AR.

Further perfusions with an alternative α_1 AR antagonist, trimazosin, were therefore undertaken. In hearts with large IZs and no added CATs, at 1 μ M, neither trimazosin nor the AR antagonists atenolol (β_1) or butoxamine (β_2), affected ischaemia-induced VF (trimazosin: 67% vs. 67% with vehicle; atenolol: 42% vs. 50% with vehicle; butoxamine: 33% vs. 56% with vehicle). In addition, none of these antagonists had any significant effects on ECG intervals (appendices figures 1, 2 and 3 A-D). At 10 μ M, however, butoxamine and trimazosin reduced VF incidence to 0% and 25%, respectively. Thus, the off target NOELs for atenolol, butoxamine and trimazosin were 1 μ M for each drug.

Identification of AR antagonist concentrations with specificity for the intended AR

At 1 μ M neither butoxamine nor atenolol had any significant effect on the dobutamine CRC ($-\log EC_{50}$ or maximum response; figure 7A-B). Butoxamine shifted the salbutamol CRC to the right in a concentration-dependent manner, with the change in $-\log EC_{50}$ reaching significance at 10 μ M (figure 7C-D). Atenolol had no significant effect on the salbutamol CRC (figure 7C) or $-\log EC_{50}$ (figure 7D). Neither butoxamine nor atenolol affected the maximum relaxation response to salbutamol (figure 7D). Trimazosin caused a concentration-dependent shift to the right of the PE CRC (figure 7E), and a significant change in the $-\log EC_{50}$ (figure 7F) without affecting the PE maximum response (figure 7F).

Thus, at 1 μ M, a lack of off-target AR-mediated effects was confirmed for all 3 drugs, and the required on-target AR specificity was confirmed for butoxamine and trimazosin. As a result, butoxamine and trimazosin, each at 1 μ M, were subsequently used in the

‘catecholamine + antagonist’ study in order to identify the AR(s) mediating the facilitation of ischaemia-induced VF by CATs in hearts with small IZs. The same concentration of atenolol was also taken forward.

Identification of AR(s) mediating facilitation of ischaemia-induced VF by CATs

The initial finding, that CATs increased ischaemia-induced VF incidence in hearts with small IZs, was reproduced in separate hearts (figure 8F). Co-perfusion of CATs with either 1 μ M atenolol (β_1 selective) or butoxamine (β_2 selective) inhibited this facilitation, whereas 1 μ M trimazosin (α_1 selective) had no effect (figure 8F). This indicates that when CATs facilitate ischaemia-induced VF this required *both* β_1 and β_2 agonism. VT facilitation was also significantly reduced by 1 μ M butoxamine, whilst 1 μ M atenolol reduced VT facilitation as a non-significant trend (figure 8E). The incidences of less severe arrhythmias were unaffected (figure 8B-D). IZ size did not vary between perfusion groups (figure 8A), the means of which were between 20-30% TVW as intended. Likewise, TVW was similar in each group (vehicle: 0.92 ± 0.01 g, CATs: 0.95 ± 0.02 g, CATs + atenolol: 0.89 ± 0.02 g, CATs + butoxamine: 0.95 ± 0.02 g, CATs + trimazosin: 0.93 ± 0.02 g).

Haemodynamic and ECG changes

Atenolol (1 μ M) reduced the CAT-induced increase in HR; butoxamine (1 μ M) had a weaker effect and trimazosin (1 μ M) had no effect (figure 9A). CAT-induced increases in coronary flow followed a similar pattern (figure 9B). Coronary ligation significantly reduced coronary flow in all groups (figure 9B). None of the antagonists prevented the sustained PR shortening or QT prolongation caused by CATs (figure 9C-D). The effects of CATs on haemodynamics and ECG intervals (figure 9A-D) reproduced initial findings (figure 9A-D).

Independent verification of β AR-specific agonism and β AR-selective antagonism

Protein phosphorylation in the UZ

CATs increased cTnI phosphorylation at Ser23/24, a downstream target of β_1 AR signalling (Puhl et al., 2016), in the UZ. This was prevented by 1 μ M atenolol but unaffected by 1 μ M butoxamine or 1 μ M trimazosin (figure 10A-B).

Western blots of the RyR2 revealed two bands (figure 10D and figure 10H), corresponding to full length RyR2 (565 kDa) and the c-terminal fragment (~400 kDa) (Li et al., 2013). The full length RyR2 protein was analysed. Phosphorylation of the RyR2 at Ser2808, a downstream target of β_2 AR-mediated $G\alpha_i$ signalling that inhibits β_1 AR-mediated RyR2 phosphorylation (Schmid et al., 2015), was increased in the UZ by 1 μ M butoxamine (figure 10C-D).

Protein phosphorylation in the IZ

In contrast to UZ data, β_1 AR signalling was evident in the IZs of vehicle controls, but although CATs tended to increase this, and atenolol blocked the increase, these trends did not reach significance (figure 10E-F). Again, in contrast to UZ data, β_2 AR signalling in the IZ appeared to be unaltered by butoxamine (figure 10G-H).

Test for IZ-mediated facilitation of ischaemia-induced VF using a low flow global ischaemia model

The method successfully achieved complete separation and regulation of flow values between low flow groups ('severe': 0.8 ml min⁻¹, 'moderate': 2 ml min⁻¹, 'mild': 4 ml min⁻¹), as was intended (figure 11C). Low flow global ischaemia caused sinus bradycardia, and this effect was not surmounted by CATs (figure 11D), although in a concurrent set of hearts with regional ischaemia (large IZs) CATs increased coronary flow and evoked sinus tachycardia (figure 11C-D), as expected. Heart mean temperature fell during ischaemia by no more than 1°C (figure 11B).

Low flow global ischaemia evoked arrhythmias (figure 11E). CATs weakly facilitated ischaemia-induced arrhythmias, but only when flow reduction was 'moderate', and importantly only late after ischaemia onset (≥ 50 minutes) and not during the phase 1 period characteristic of regionally ischaemic hearts; moreover the effect obtained statistical significance only for arrhythmia score (figure 11F). QT interval was prolonged by catecholamines, but only in the 'moderate' flow reduction group (figure 11F-G).

Discussion

Overview

Published studies do not provide a clear description of the relationship between CATs, derived from the circulation or released by cardiac sympathetic innervation, and ischaemia-related arrhythmogenesis, with some findings indicative of a link (Meredith et al., 1991; Slavikova et al., 2007) and others indicative of no link (Botting et al., 1983; Curtis et al., 1985; Daugherty et al., 1986; Paletta et al., 1989). The rat Langendorff preparation is devoid of autonomic nervous reflex or circulating CATs, yet ischaemia- or reperfusion-induced VF normally occurs in almost every heart when the IZ is large, meaning that CATs are *not necessary mediators* of ischaemia-induced VF (Ridley et al., 1992; Wilder et al., 2016). The aims of this study were therefore (i) to examine the hypothesis that CATs *facilitate* (rather than mediate) ventricular arrhythmias caused by acute myocardial ischaemia, and (ii) to identify the ARs involved. When IZ was made small, ischaemia-induced VF was found to be facilitated by a mixture of CATs that mimics the cardiac effects of sympathetic tone by restoring HR to levels seen in rats *in vivo* (Curtis et al., 1985) and reducing PR interval, as found previously (Clements-Jewery et al., 2002). VF facilitation was not detectable with larger IZs. Facilitation was inhibited by atenolol (β_1) and butoxamine (β_2), but not trimazosin (α_1), with haemodynamic, western blot and ancillary vascular bioassay data confirming antagonist specificity and selectivity at the concentrations used. The findings show that, when there is *scope*, exogenous CATs can increase the risk of ischaemia-induced VF. *Scope* requires the intrinsic risk of VF to be low relative to that occurring with, for example, a large IZ of 40% or more of ventricular mass. These data, and the findings from hearts subjected to low flow global ischaemia (no VF facilitation during the 'phase 1' period of early ischaemia), indicate an action mediated primarily if not exclusively in the UZ. The antagonist data revealed the effects to be to β_1 and β_2 AR-mediated, confirmed by western blotting, with activation of both receptors necessary for facilitation.

Role of alterations in HR, coronary flow and QT in mediating the facilitation of VF

In rat isolated hearts with large IZs (~40% TVW) there is a relationship between HR and ischaemia-induced VF, but this becomes evident only at HRs in the sinus tachycardia range (e.g. 480 beats min⁻¹) (Bernier et al., 1989). In dogs *in vivo*, likewise, variation in HR over the normal range for the species (i.e. 150-200 beats min⁻¹) had little effect on ischaemia-induced

VF risk (Bolli et al., 1986). In the present study, in hearts paced to match the HR of CAT-perfused hearts ($405 \text{ beats min}^{-1}$), there was no increase in ischaemia-induced VF incidence, meaning that VF facilitation by CATs was not secondary to sinus tachycardia.

CAT perfusion increased QT interval in the catecholamines + antagonist study (figure 8D), as found previously (Clements-Jewery et al., 2002; Clements-Jewery et al., 2006). In previous studies, CATs were introduced 90 minutes after the onset of regional ischaemia to hearts that had, by this time, recovered sinus rhythm following the paroxysms of early (phase 1) arrhythmias (Clements-Jewery et al., 2002). At this time, rat hearts are susceptible to developing phase 2 arrhythmias, which manifest primarily by non-re-entrant mechanisms (Clements-Jewery et al., 2009), different from the flow of injury current and re-entry that operate during the first 30 minutes of ischaemia (phase 1) (Sidorov et al., 2011). CAT perfusion failed to facilitate phase 2 arrhythmias (Clements-Jewery et al., 2002; Clements-Jewery et al., 2006), despite the fact that the (predominantly non-re-entrant) mechanisms of phase 2 are supposedly highly susceptible to facilitation by CATs (Antzelevitch & Burashnikov, 2011). If the QT prolongation observed in the present study were to have had any effect on the re-entrant mechanisms occurring during phase 1, *suppression* of VF rather than facilitation would be the expected outcome (Nattel, 2008). Thus, it is not tenable that the observed QT prolongation contributed to the facilitation of ischaemia-induced VF by CATs. It should be noted that the lack of identifiable QT interval prolongation by CATs in the varied IZ size study (figure 3D) was likely due to differences in baseline (pre-ischaemia/pre-switch) QT interval compared to baseline QT interval in the catecholamines + antagonist study (figure 8D), meaning that there was reduced scope for detection of CAT-induced prolongation. In both studies, QT interval was approximately 80 ms after switching to CATs.

Evidence of drug selectivity and specificity for AR identification

In order to identify the ARs mediating the CAT effects, it was necessary to select concentrations of AR antagonists that exhibited selectivity and specificity. Specificity was established from rat aorta myography experiments and/or from evidence of antagonism of haemodynamic and intracellular signalling effects of CATs in perfused hearts. Selectivity was established from a lack of actions indicative of non-selectivity, in particular, a lack of effect on ischaemia-induced VF in hearts perfused without added CATs.

Prazosin and terazosin both failed to meet specificity/selectivity requirements. The mechanism of their 'off target' effects is irrelevant in the present context, albeit prazosin certainly possesses Na^+ channel blocking activity (Daugherty et al., 1986; Thandroyen et al., 1983). In contrast, atenolol, butoxamine and trimazosin met the selectivity requirements at 1 μM . This does not necessarily mean that these antagonists do not have any AR-independent actions at 1 μM , but it shows that any such actions do not affect any of the haemodynamic and ECG variables measured, or inhibit ischaemia-induced arrhythmias in the absence of exogenous CATs. Furthermore, 1 μM of these drugs also possessed the required AR specificity, judged from ancillary whole heart pharmacology (e.g., effects on HR), ancillary rat aorta myography experiments, and/or western blotting. The myography showed that 1 μM butoxamine and 1 μM trimazosin had the necessary specificity for β_2 or α_1 ARs. The specificity of atenolol for the β_1 AR, however, could not be established from the aorta assay. This was unanticipated, but we found, after completing studies that the rat thoracic aortae has a low expression of β_1 ARs (Perez-Aso et al., 2014). Specificity of atenolol for the β_1 AR was nevertheless established independently from its ability to antagonise the increase in HR caused by CATs (figure 9A) and from the abolition of the CAT-stimulated β_1 AR signal (p-cTnI/cTnI; figure 10A). The array of effects on multiple variables therefore confirmed the specificity and selectivity of 1 μM of butoxamine, trimazosin and atenolol, establishing their validity as pharmacological tools in the present context.

Facilitation of ischaemia-induced VF via actions in the IZ

Collateral flow in the rat IZ is <5% normal flow (Maxwell et al., 1987) and the ischaemic milieu in the rat heart is effectively stagnant and not conducive to delivery and accumulation of labile substances such as CATs. However should sufficient amounts of CATs be able to access the IZ during early ischaemia, as may occur in well-collateralised hearts of some species other than rat, AR activation in the IZ may be sufficient to facilitate ischaemia-induced arrhythmias. To test this, a low flow global ischaemia model was used, in which the site of action of perfused CATs is, unavoidably and exclusively, the IZ, and residual flow can be controlled precisely. CATs did facilitate ischaemia-induced arrhythmias in these hearts, but effects were weak, detectable only by using an arrhythmia score that (unlike VF incidence) permits use of powerful parametric statistics, and required a prolonged period of ischaemia to become apparent (well beyond the window of phase 1 arrhythmias associated

with *regional* ischaemia) (Curtis, 1998). Furthermore facilitation occurred only when coronary flow was reduced ‘moderately’ to 2 ml min^{-1} , and not when flow reduction was mild or severe, consistent with a need for residual flow to be sufficiently high to deliver CATs swiftly enough to preclude the perfused tissue degrading them, yet sufficiently low to cause ischaemia of necessary severity to generate an arrhythmogenic substrate. Overall, the weak facilitation of ischaemia-induced VF by CATs acting within the IZ was insufficient, and far too delayed, to have contributed meaningfully to the facilitation of VF observed during regional ischaemia.

There is evidence that endogenous NA may accumulate in the ischaemic region owing to local acidity driving uptake 1 in the reverse mode in the surviving sympathetic afferents, and that this occurs even in rat isolated hearts in which the postganglionic sympathetic afferents are unavoidably detached from the CNS (Schömig et al., 1984). However this process is unlikely to have contributed to any of the present study outcomes. There are several reasons for this.

The first is that the key variable we examined was the *increase* in VF incidence caused by perfusion with catecholamines delivered to the uninvolved *non-ischaemic* region. It is difficult to conceptualise how uptake 1 in the reverse mode in the ischaemic region could be increased by perfusing the uninvolved region with catecholamines, or how this, if it occurs, would account for the increase in ischaemia-induced VF caused by perfusion with catecholamines. To examine the receptor mechanism we used AR antagonists shown to have no action on ischaemia-induced VF in hearts perfused *without* added catecholamines (meaning these drugs had no effect on the actions of any *endogenous* NA released via uptake 1 in the reverse mode during ischaemia). Likewise, it is not possible to explain inhibition of the increase in ischaemia-induced VF caused by perfusion with catecholamines by the two β AR antagonists (butoxamine and atenolol at $1 \mu\text{M}$) on the basis of a supposed inhibition of the actions of endogenous NA in the ischaemic region (released by uptake 1 in the reverse mode) because these drugs at the concentration used ($1 \mu\text{M}$) had no effect on ischaemia-induced VF incidence in hearts perfused *without* added catecholamines.

Whether or not NA released by uptake 1 in the reverse mode can contribute to ischaemia-induced VF in a wider sense is a separate issue that may warrant separate investigation. In view of possible reader interest in the topic, we have expanded on the role of uptake 1 in ischaemia-induced VF below.

Interestingly, the data derived by Schömig et al. (1984) do not show that uptake 1 in the reverse mode operates *during ischaemia*. Schömig et al. (1984) measured NA release during *reperfusion*. Other investigators have made similar measurements, again during reperfusion (Wilde et al., 1988). One of the few papers that measured NA within ischaemic tissue itself found a *reduction* in tissue NA content in a rat *in vivo* model after 20 and 30 minutes of ischaemia (earlier time points not examined) (Abrahamsson et al., 1981). The latter study refers to the conflicting evidence for changes in tissue NA during ischaemia. There is evidence that NA accumulates in the interstitial fluid in the ischaemic myocardium from work by Lameris et al. (2000) using microdialysis probes implanted into the left ventricle of pigs. But just as in the case of Schomig's rat heart data (Schömig et al., 1984), the time course of accumulation shows a large with the known temporal pattern of VF during ischaemia; interstitial NA was still rising 60 minutes after the onset of ischaemia, long after the phase 1 arrhythmia period is over in pigs, while values at the peak of arrhythmia incidence were relatively low (Lameris et al., 2000).

Species differences, or experimental setting, may also be an issue. The study by Lameris et al. (2000) shows that ischaemia causes release of NA in pigs *in vivo*. However, as noted above, the evidence for this in isolated denervated rat hearts is less persuasive. Indeed, Daugherty et al. (1986) found that during continuous regional ischaemia in the rat Langendorff preparation (with no added catecholamines), the low level of collateral flow allowed the release of accumulating lactate to be detected during ischaemia, but there was no commensurate release of NA. This confirms that the release of NA during reperfusion observed by Schömig et al. (1984) in the rat Langendorff was, in part at least, *reperfusion-induced*, rather than washout of NA that had accumulated during ischaemia.

There is actually very little work that addresses specifically the role of uptake 1 in the reverse mode as an arrhythmogenic mechanism. Few of the published studies measure arrhythmias and NA contiguously. In one of the more recent relevant articles published on the subject, (Du et al., 1998), the authors noted "We found that depletion of noradrenaline prevented arrhythmias in anoxic hearts but failed to do so in ischemic hearts" and added that "in noradrenaline-depleted hearts, desipramine and imipramine . . . remained potent in preventing arrhythmias". Ischaemia-induced VF can therefore still occur in hearts depleted of catecholamines, and this is inhibited by drugs that inhibit uptake 1, meaning there can be no obligatory or necessary role for uptake 1 in the reverse mode in the mediation of ischaemia-

induced VF, and that the drug tools used earlier by Schömig et al. (1984), and others (Kurz et al., 1995; Richardt et al., 2006) evidently lack selectivity for uptake 1.

Overall, these data show there is no basis for giving serious consideration to the possibility that uptake 1 in the reverse mode has relevance to the present study's findings.

Identification of the ARs mediating facilitation of VF

Given the selectivity and specificity of 1 μ M atenolol and butoxamine, their effects on VF indicates, somewhat unexpectedly, that simultaneous activation of β_1 and β_2 ARs is necessary for VF facilitation by CATs since, if activation of only one or other AR were necessary, only one or other of the antagonists would have had the capability to reduce VF incidence.

With respect to mechanism, separate pacing studies showed that VF facilitation was not a secondary consequence of an increase in HR, and inhibition of VF facilitation was not secondary to any inhibition of the increase in HR.

In UZs, atenolol prevented the phosphorylation of cTnI by CATs, indicative of inhibition of β_1 AR signalling (Puhl et al., 2016), whereas butoxamine and trimazosin had no effect. These data confirmed the evidence outlined above that atenolol inhibited VF facilitation via β_1 antagonism whereas butoxamine inhibited VF facilitation via a β_1 -independent action.

Evidence of suppression of β_2 AR mediated signalling by butoxamine was sought by probing for RyR2 phosphorylation, a relatively novel and unestablished method that depends upon β_1 AR signalling increasing RyR2 phosphorylation (via $G\alpha_s$), whilst β_2 AR signalling attenuates it (via $G\alpha_i$) (Schmid et al., 2015). The, p-RyR2 levels in the UZ were increased by CATs + butoxamine vs. vehicle-perfused hearts, as anticipated. However, p-RyR2 levels were unchanged in the CATs + atenolol group, which would suggest that the RyR2 is phosphorylated additionally by AR-independent mechanisms. This would also explain the detectable baseline levels of p-RyR2 in the UZs of vehicle-perfused hearts, and lack of detectable attenuation of p-RyR2 in CAT + atenolol-perfused hearts in the present study. The β_2 AR signalling assessment was therefore disappointing. Nevertheless, the key finding was that butoxamine (but not atenolol) demonstrated specificity at the β_2 AR. Taken together, the present data confirm that butoxamine reduced the facilitation of ischaemia-induced VF by CATs via selective and specific β_2 AR antagonism.

Although we processed IZ tissue, the data were shown for purposes of transparency rather than to explore any hypothesis. The IZ western blots were more variable than blots from the UZs, as one might expect given that ischaemia as well as CATs can affect AR signal expression levels. For example, cTnI phosphorylation at Ser23/24 is elevated during regional ischaemia in murine hearts (Nixon et al., 2014), and the acidic conditions of ischaemia can increase p-cTnI in rat hearts (Mundina-Weilenmann et al., 1996).

Nevertheless, atenolol and butoxamine had no effect on β_1 or β_2 signalling in the IZ, consistent with the more compelling evidence discussed above that the facilitation of VF by CATs and its inhibition by atenolol or butoxamine were mediated primarily if not exclusively in the UZ.

Mechanistic insight and limitations of present findings

It was not our objective to explore the electrophysiological mechanisms by which CATs facilitate ischaemia-induced VF. This is partly because it is well known that ischaemia-induced changes in IZ AP shape and conduction velocity that generate AP heterogeneity between the IZ and UZ, and facilitate arrhythmogenic flow of injury current (Janse et al., 1980; Janse & Wit, 1989) are exacerbated by CATs via actions in the UZ (the AP depolarises faster, increasing conduction velocity, and hyperpolarises to a more negative membrane potential during diastole) (Dorian, 2005; Janse & Wit, 1989). What was not known, and what formed the focus of this study, was the extent to which these effects of CATs contributes to ischaemia-induced VF. The mechanistic insight provided by the present data concerns the identification of the scope for VF facilitation by CATs (i.e., the potential relevance), the site of action and the identity of the receptor mechanisms responsible (which are targetable by drugs).

As in previous studies (Clements-Jewery et al., 2002; Clements-Jewery et al., 2006), our method of mimicking sympathetic influence in a preparation that ordinarily has none is a practicable and tractable approach, and one that is validated in terms of achievement of what is intended – restoration of cardiac variables to levels typical of the conscious rat (Clements-Jewery et al., 2002). However, perfusion with any mix of NA and AD cannot mimic precisely the influence of the sympathetic nervous system on the heart *in vivo*, which comprises a mix of circulating CATs and noradrenergic neurotransmission, neither of which are ever in true

steady state, meaning the possibility of additional VF-facilitating actions secondary to autonomic turbulence. Modelling this may be warranted in future studies.

Conclusion

In rat isolated perfused hearts in which baseline risk of phase 1 ischaemia-induced VF was made deliberately low, exogenous CATs facilitated VF with effects mediated predominantly in the UZ. The facilitation occurred via β_1 and β_2 agonism, with activation of both types of AR necessary. These findings may partially explain why, in humans, β blockers (of varying selectivity) have only moderate benefit in reducing mortality and ventricular arrhythmias in patients at risk of acute MI (β -Blocker Heart Attack Study Group, 1981), since VF risk is likely to be reduced only in patients with smaller IZs.

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Tables**Table 1:** Intended IZ size

Position of Ligature	IZ size	% TVW
Distal	Small	<20
Medial	Medium	25-35
Proximal	Large	>42

For Peer Review

Figure Legends

Figure 1: The 5 types of ventricular arrhythmias occurring during ischaemia and reperfusion in Langendorff-perfused rat hearts, as defined by the Lambeth Conventions II (Curtis et al., 2013). **VPB:** ventricular premature beat, **BG:** bigeminy, **VT:** ventricular tachycardia, **VF:** ventricular fibrillation.

Figure 2: Experimental protocols for varied IZ size study, pacing study, antagonist only studies, catecholamines + antagonist study and low flow global ischaemia study. **IZ:** ischaemic zone, **CATs:** catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 3: IZ sizes resulting from distal, medial or proximal ligation of the left coronary artery (A) and incidence (% of group) of increasingly severe ventricular arrhythmias (VPB to VF) during 30 minutes of regional ischaemia (B-F) in hearts with small, medium and large IZs (varied IZ size study). $n = 20$ per group; mean \pm SEM (A) or incidence (B-F); * $p < 0.05$. **TVW:** total ventricular weight, **IZ:** ischaemic zone, **VPB:** ventricular premature beat, **BG:** bigeminy, **VT:** ventricular tachycardia, **VF:** ventricular fibrillation, **CATs:** catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 4: Haemodynamic and ECG changes in hearts with small, medium and large IZs (varied IZ size study). A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 20$ per group; mean \pm SEM; * $p < 0.05$ all CATs groups vs. time-matched corresponding control groups; † $p < 0.05$ small IZ control vs. time-matched small IZ + CATs; # $p < 0.05$ medium IZ control vs. time-matched medium IZ + CATs; § $p < 0.05$ large IZ control vs. time-matched large IZ + CATs. **HR:** heart rate, **PR:** PR interval, **QT:** QT₉₀ interval, **CATs:** catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 5: Incidence (% of group) of increasingly severe ventricular arrhythmias (VPB to VT) during 30 minutes of regional ischaemia and 1 minute of reperfusion in hearts with small IZs, with or without left atrial pacing (pacing study). $n = 12$ per group (ischaemia) or 6 per group (reperfusion). N.B. the incidence of ischaemia- and reperfusion-induced VF was zero in both groups. **VPB:** ventricular premature beat, **BG:** bigeminy, **VT:** ventricular tachycardia.

Figure 6: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μ M prazosin (A-D), and 1 or 10 μ M terazosin (E-H). HR (A+E), coronary flow (B+F), PR (C+G) and QT intervals (D+H). $n = 12$ per group; mean \pm SEM; * $p < 0.05$ vs. time-matched vehicle. **HR:** heart rate, **PR:** PR interval, **QT:** QT₉₀ interval.

Figure 7: Dobutamine (A), salbutamol (C) and PE (E) CRCs in the presence of vehicle, 1 or 10 μ M atenolol, butoxamine or trimazosin, and $-\log EC_{50}$ s and maximum response values (B, D, F) derived from the CRCs. $n = 5$ per group; mean \pm SEMs; * $p < 0.05$. **CRCs:** concentration response curves, **PE:** phenylephrine.

Figure 8: IZ size (A) and incidence of increasingly severe ventricular arrhythmias (VPB to VF; B-D) during 30 minutes of regional ischaemia in hearts with small IZs, perfused with vehicle, CATs or CATs + 1 μ M atenolol,

butoxamine or trimazosin. $n = 20$ per group; mean \pm SEM (A) or incidence (B-F); * $p < 0.05$. **TVW**: total ventricular weight, **IZ**: ischaemic zone, **VPB**: ventricular premature beat, **BG**: bigeminy, **VT**: ventricular tachycardia, **VF**: ventricular fibrillation, **CATs**: catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 9: Haemodynamic and ECG changes in hearts with small IZs perfused with vehicle, CATs or CATs + 1 μ M atenolol, butoxamine or trimazosin. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 20$ per group; mean \pm SEM; $p < 0.05$ indicated in the figure. **HR**: heart rate, **PR**: PR interval, **QT**: QT₉₀ interval, **CATs**: catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 10: Quantification of UZ (A-D) and IZ (E-H) western blots probed for total cTnI and p-cTnI (A-B, E-F), and total RyR2 and p-RyR2 (C-D, G-H). $n = 5$ per group; mean \pm SEM; * $p < 0.05$. **p-cTnI**: phosphorylated cardiac troponin I, **Casq2**: calsequestrin 2 (cardiac), **p-RyR2**: phosphorylated ryanodine receptor type 2 (cardiac), **CATs**: catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 11: IZ size in hearts with large IZs (A), heart temperature (B), coronary flow (C), HR (D), mean 5 point arrhythmia score in hearts perfused without CATs (E), 5 point arrhythmia score from hearts with 'moderate' (2 ml min⁻¹) low flow global ischaemia (F), and QT interval at 60 minutes of ischaemia (G) in hearts with low flow global ischaemia or large IZs, perfused with vehicle or CATs. $n = 12$ per group; mean \pm SEM; † $p < 0.05$ vs. time-matched large IZ vehicle control; * $p < 0.05$. **HR**: heart rate, **QT**: QT₉₀ interval, **CATs**: catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Appendices Figure 1: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μ M atenolol. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 12$ per group; mean \pm SEM; * $p < 0.05$ vs. time-matched vehicle. **HR**: heart rate, **PR**: PR interval, **QT**: QT₉₀ interval.

Appendices Figure 2: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μ M butoxamine. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 12$ per group; mean \pm SEM; * $p < 0.05$ vs. time-matched vehicle. **HR**: heart rate, **PR**: PR interval, **QT**: QT₉₀ interval.

Appendices Figure 3: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μ M trimazosin. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 12$ per group; mean \pm SEM. **HR**: heart rate, **PR**: PR interval, **QT**: QT₉₀ interval.

Appendices Figure 4: Relationship between TVW and VF incidence from all hearts used in all studies. TVW: total ventricular weight, VF: ventricular fibrillation. Mean \pm SEM, Pearson correlation calculation.

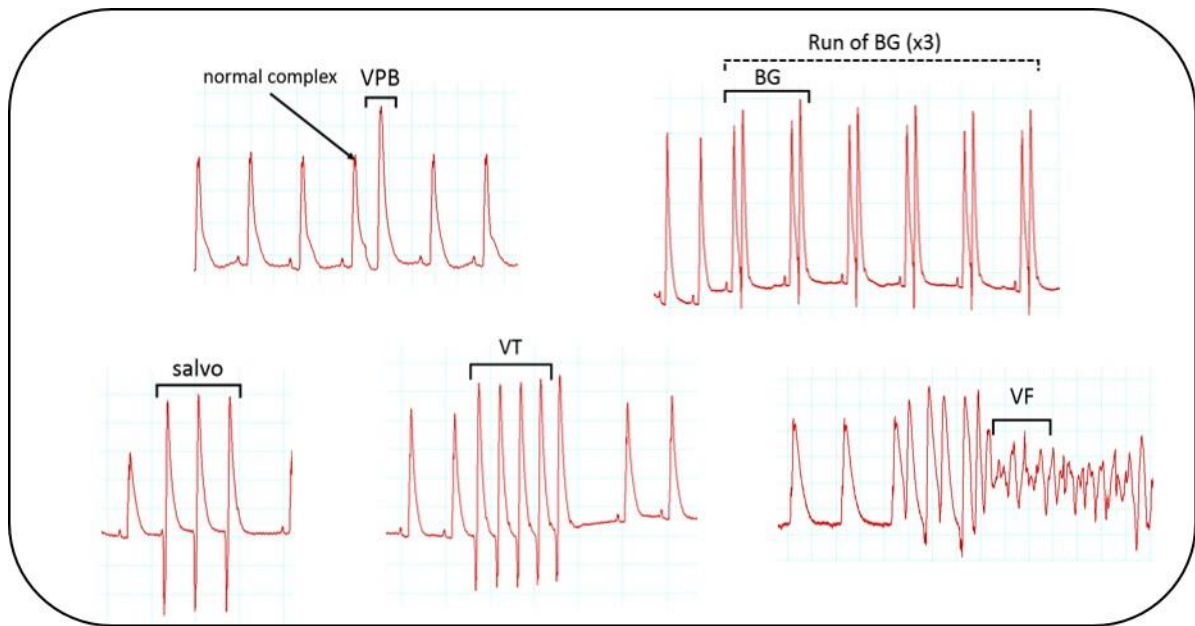
Figure 1

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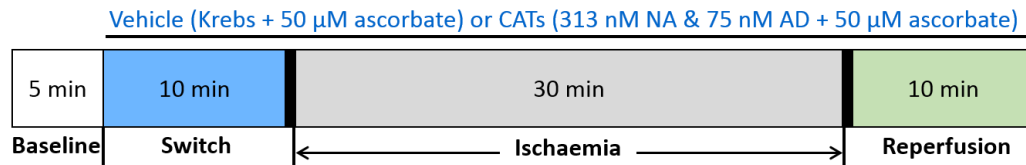
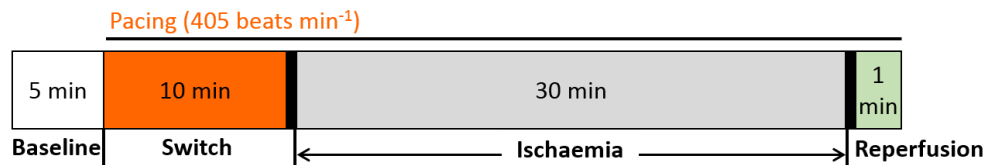
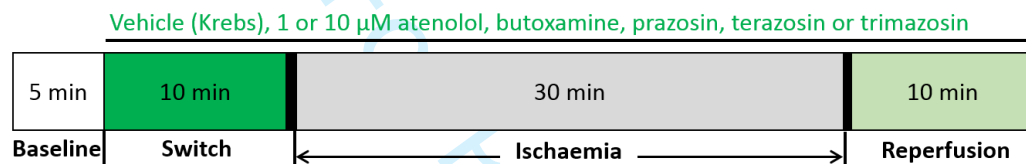
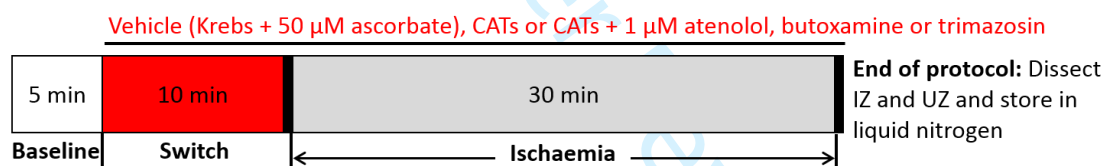
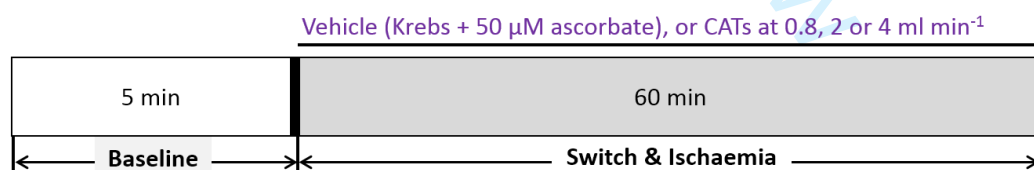
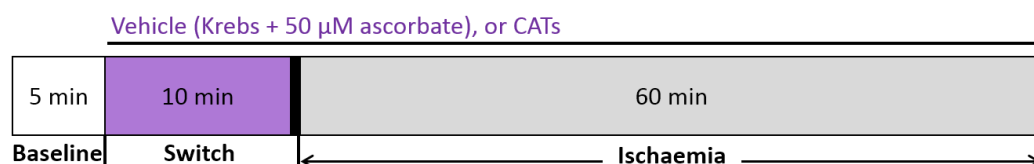
Figure 2**Varied IZ size study (small, medium or large IZ)****Pacing study (small IZ)****Antagonist only studies (large IZ)****Catecholamines + antagonist study (small-medium IZ)****Low flow global ischaemia study:****1. low flow global ischaemia: 0.8, 2 or 4 ml min^{-1}** **2. regional ischaemia: large IZ**

Figure 2: Experimental protocols for varied IZ size study, pacing study, antagonist only studies, catecholamines + antagonist study and low flow global ischaemia study. **IZ:** ischaemic zone, **CATs:** catecholamine mixture (313 nM noradrenaline + 75 nM adrenaline).

Figure 3

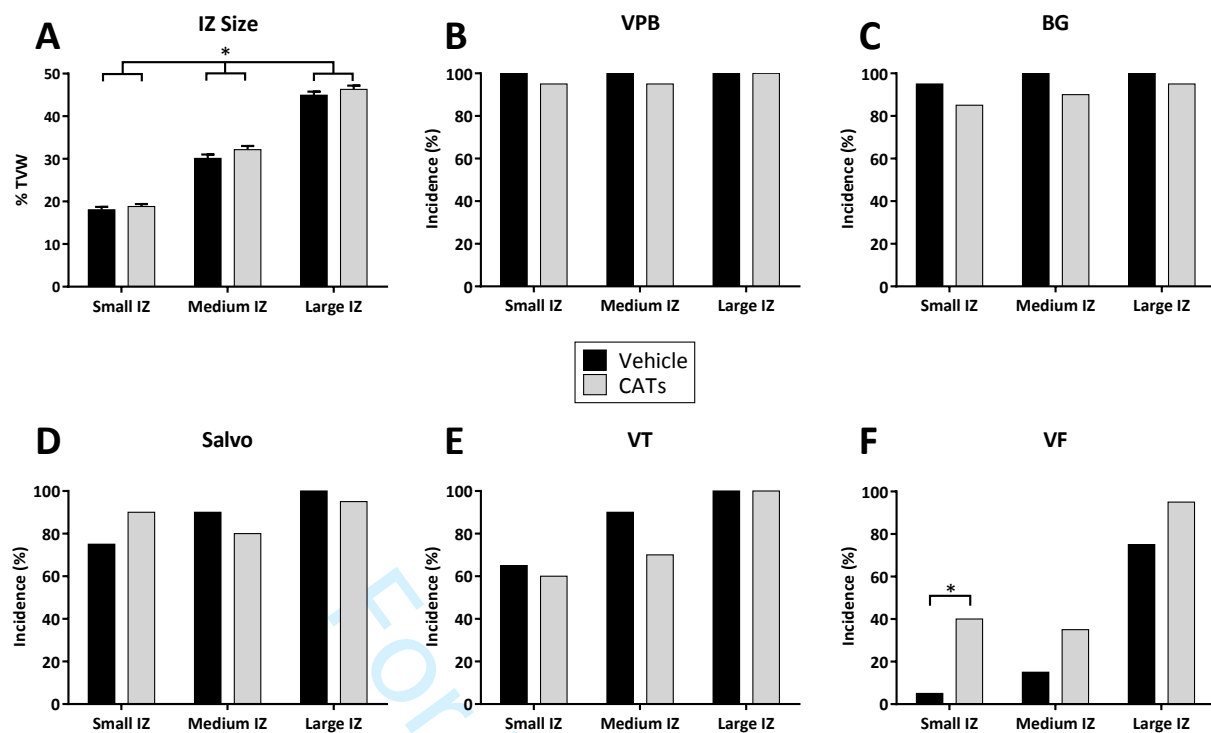


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Figure 4

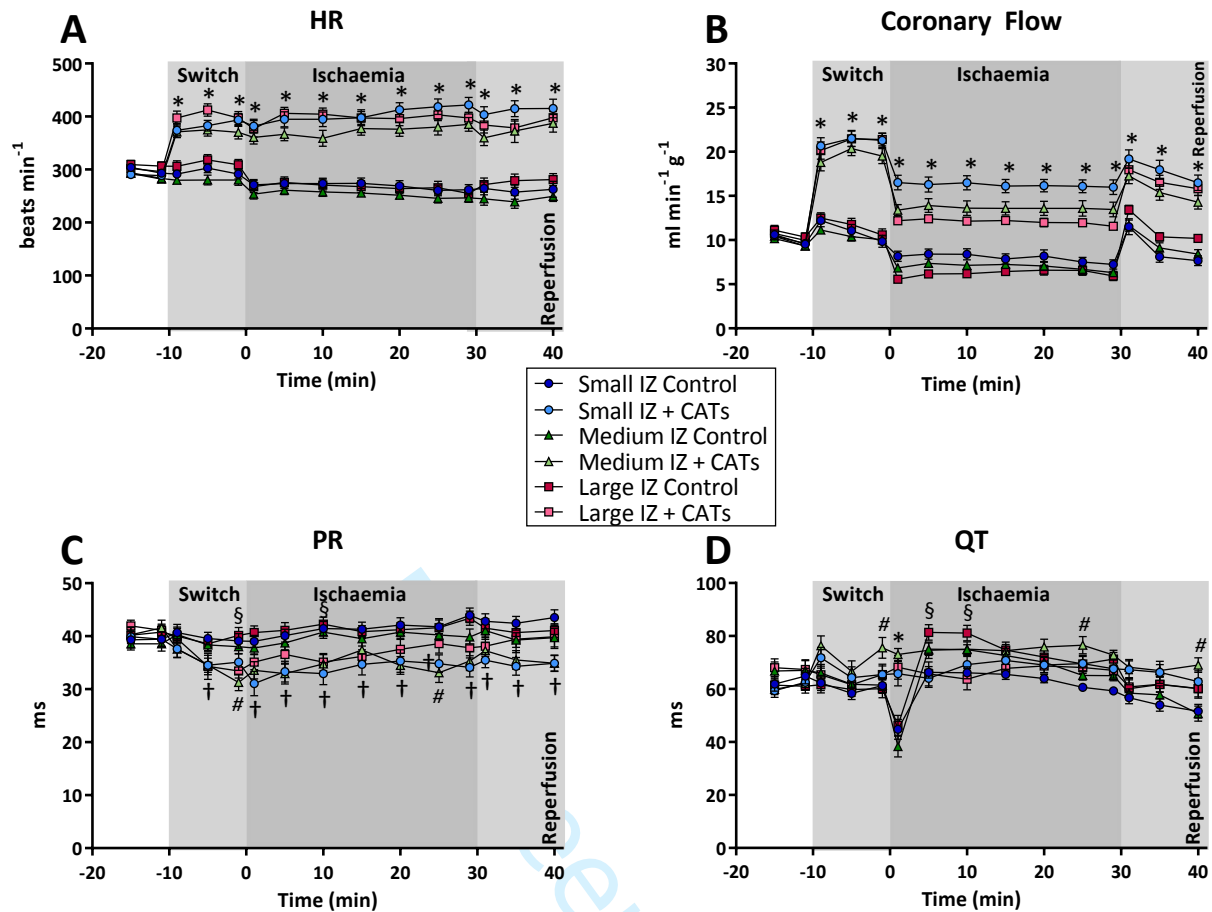


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Figure 5

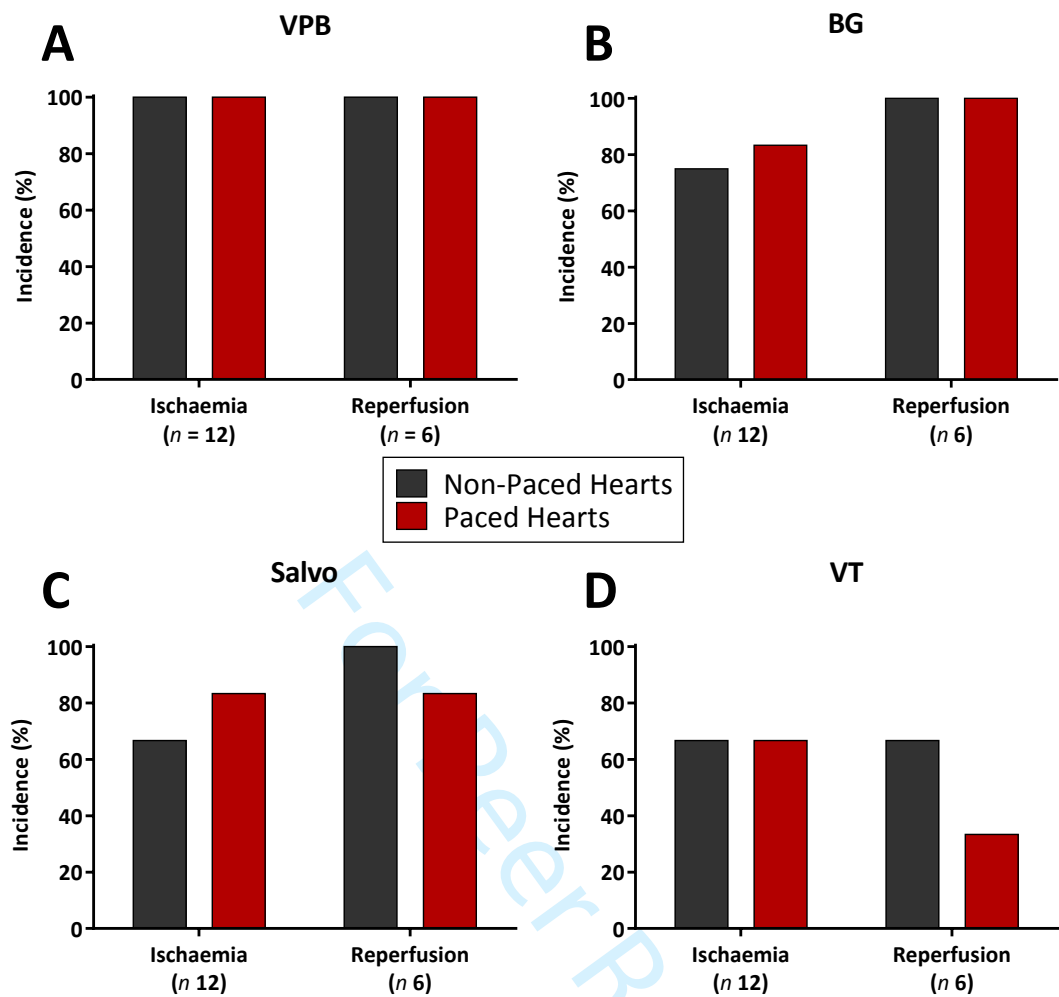


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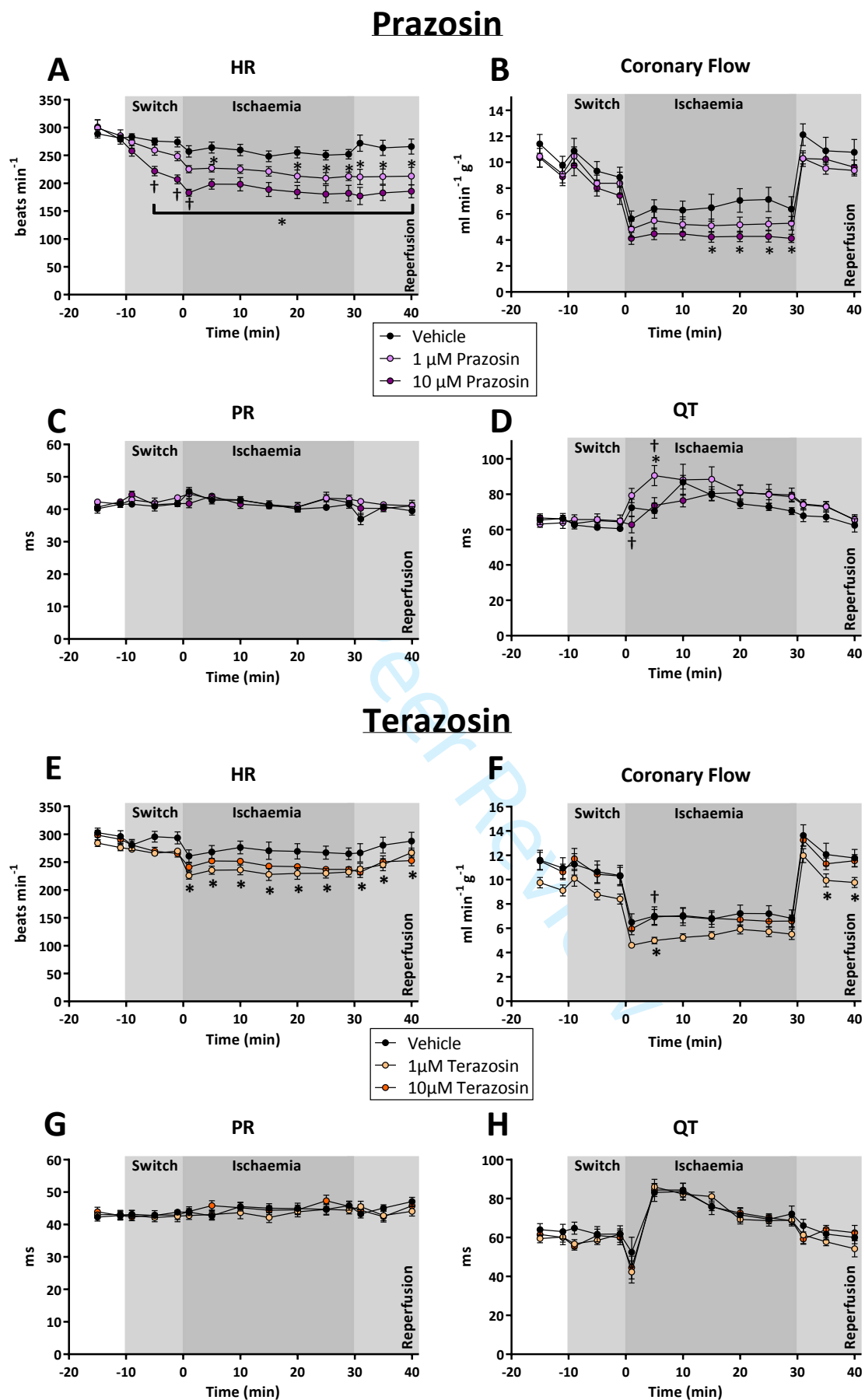


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Figure 7

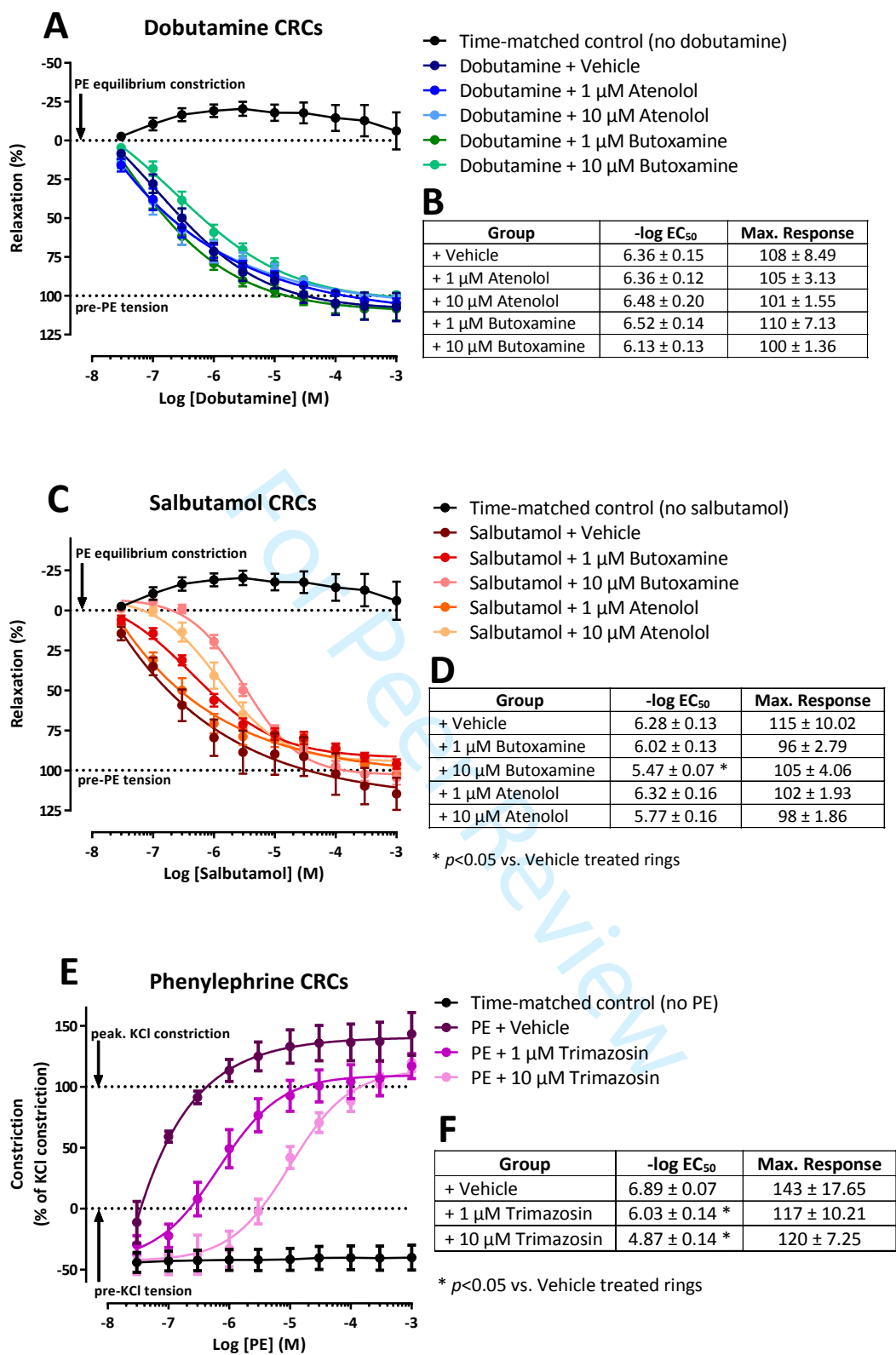


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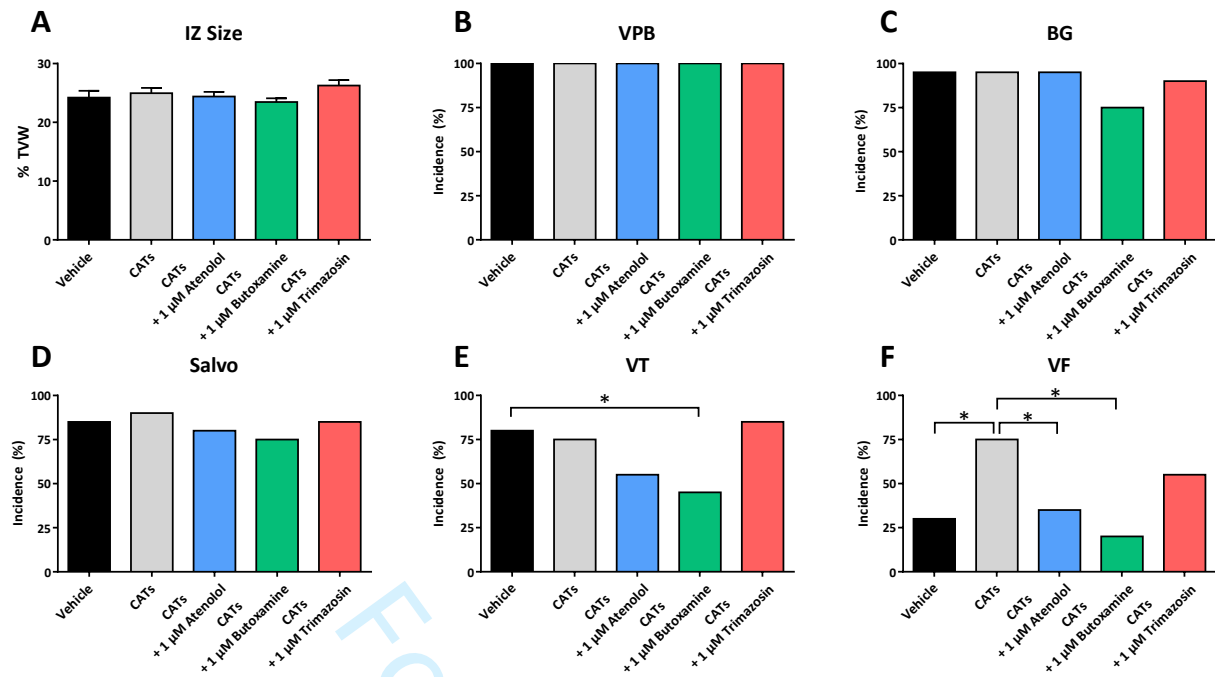
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Figure 9

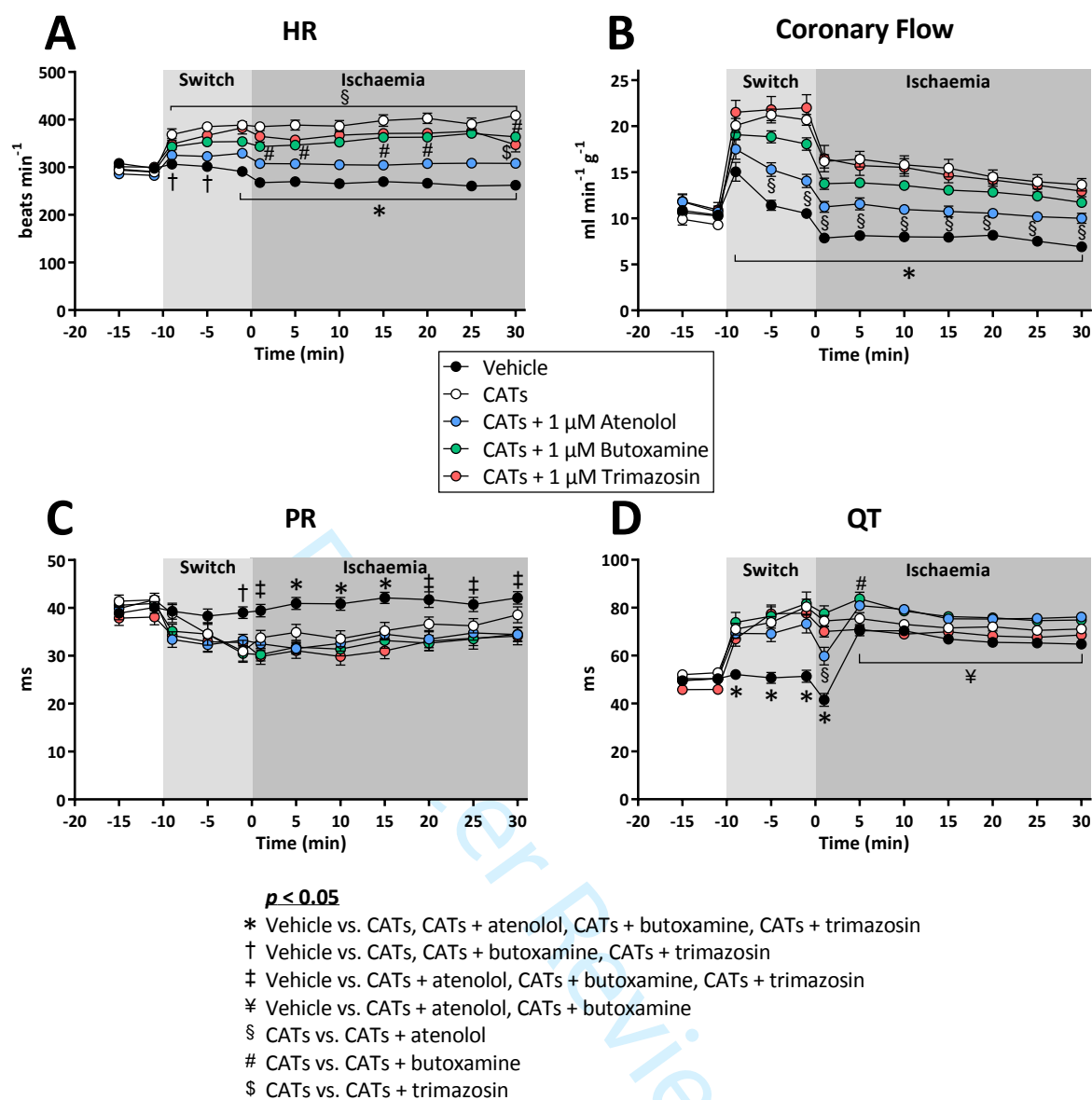


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Figure 10

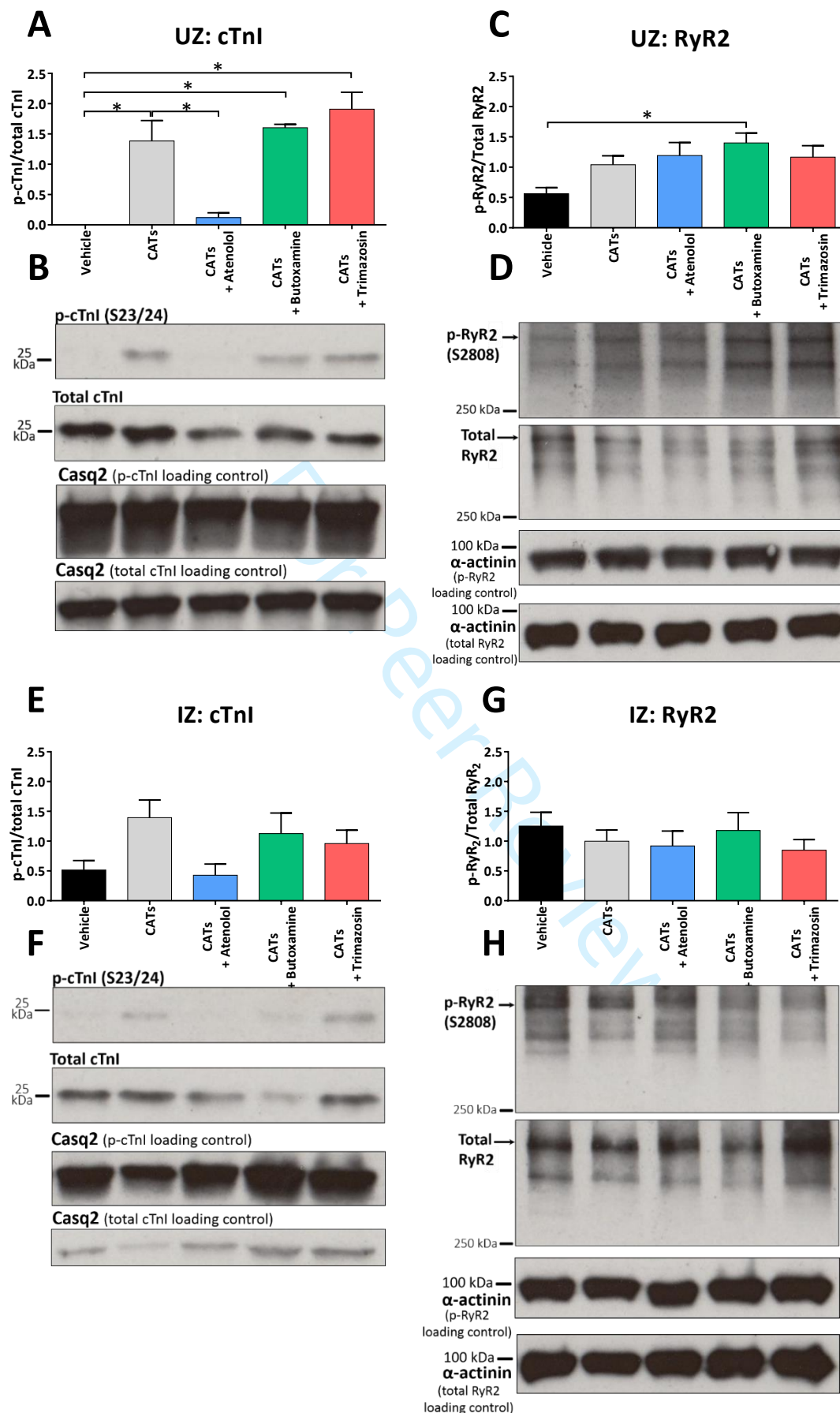


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Figure 11

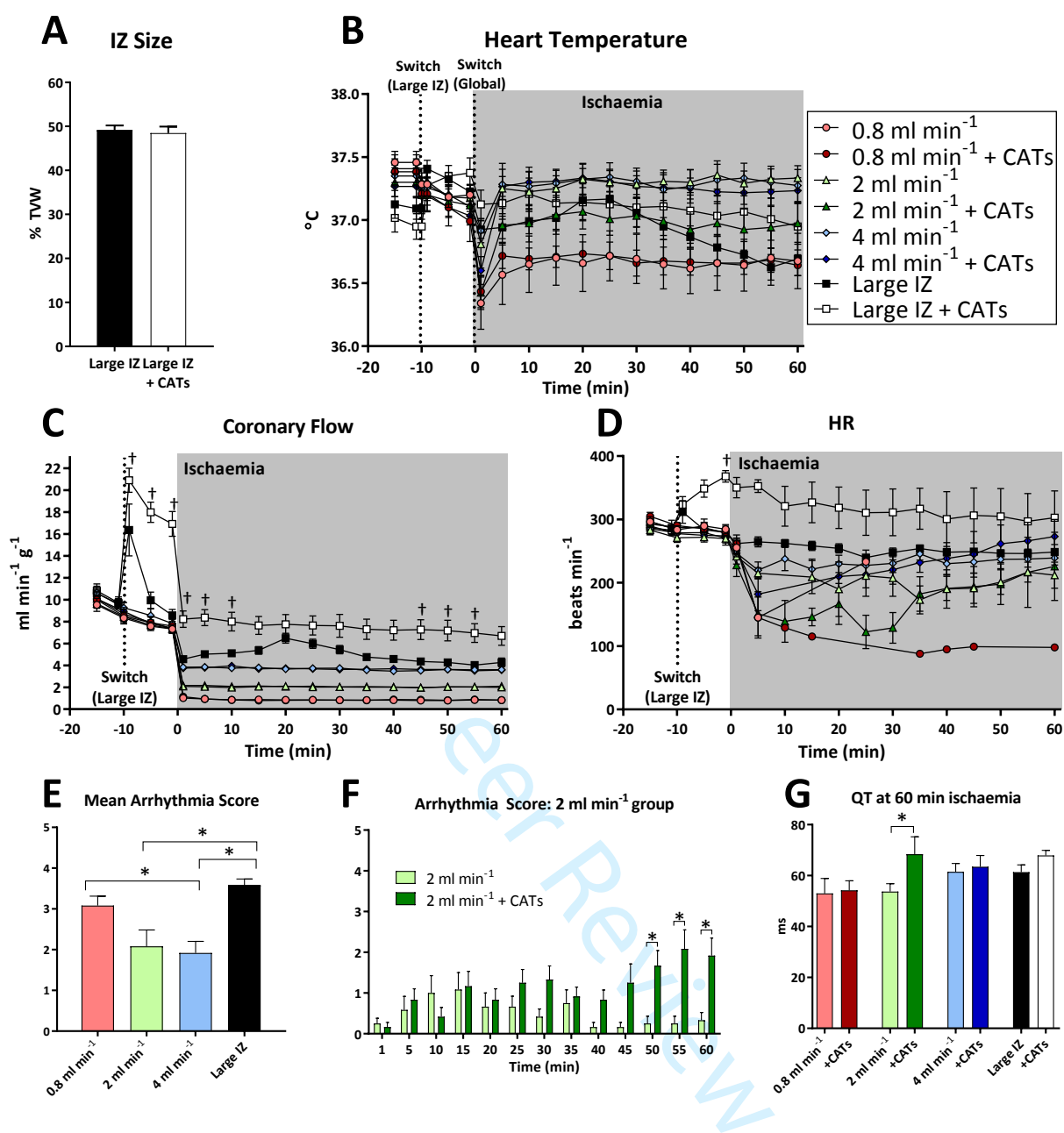
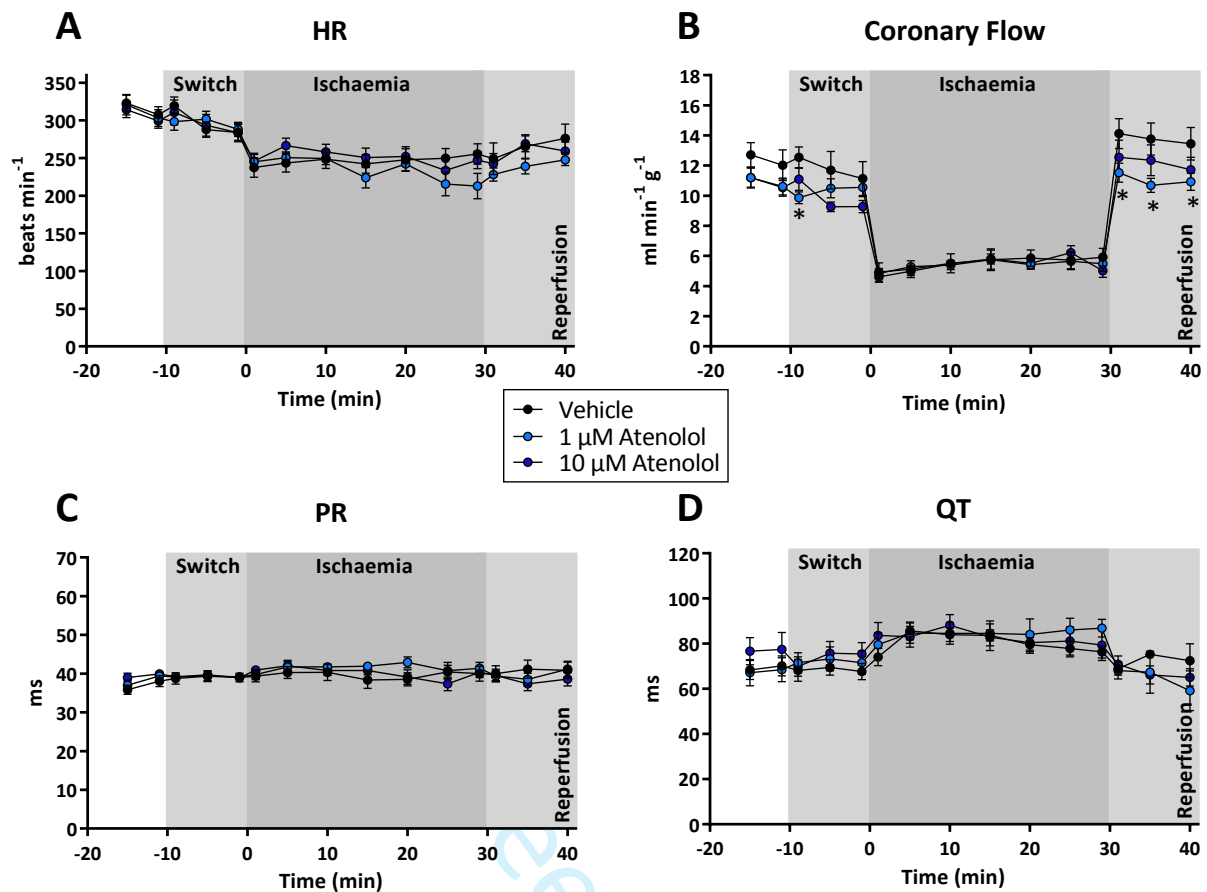


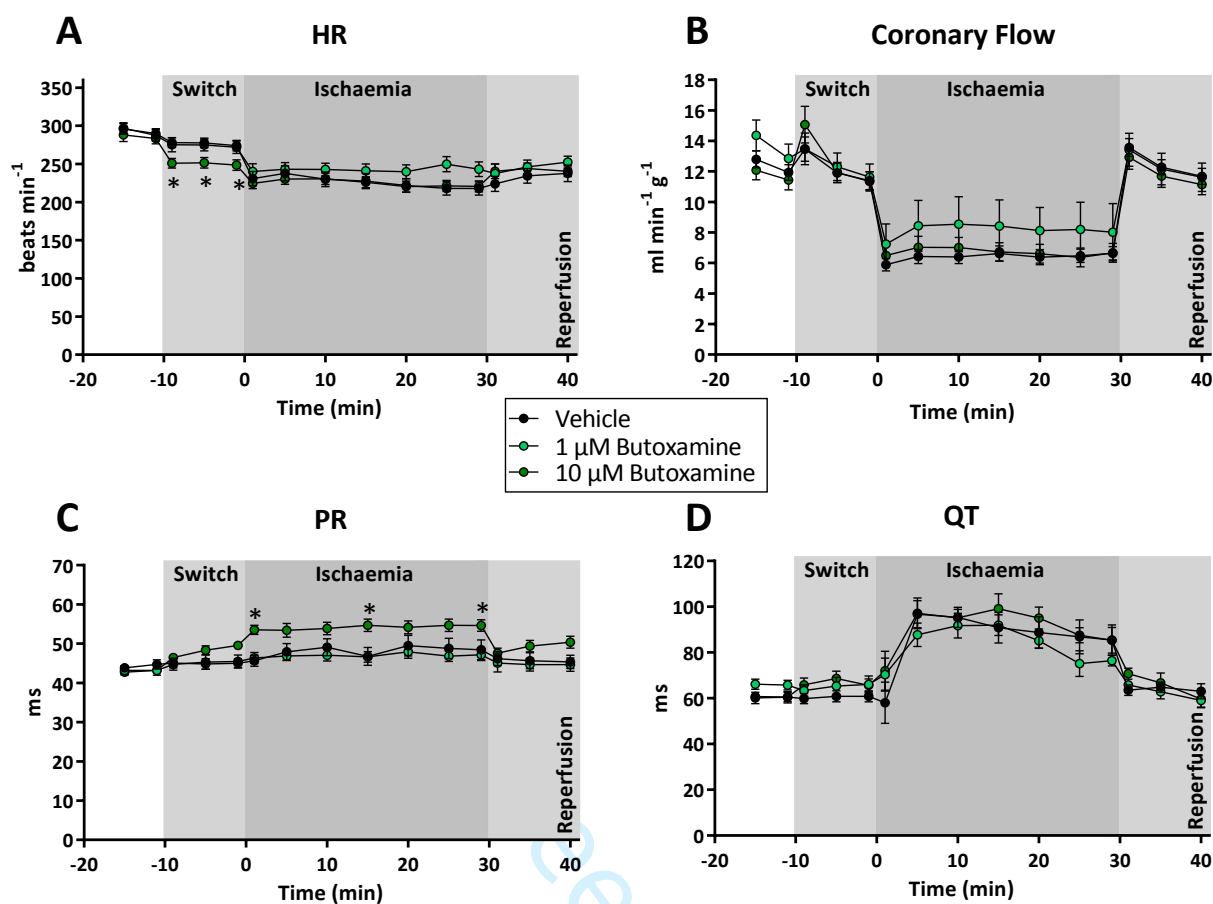
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Appendices Figure 1



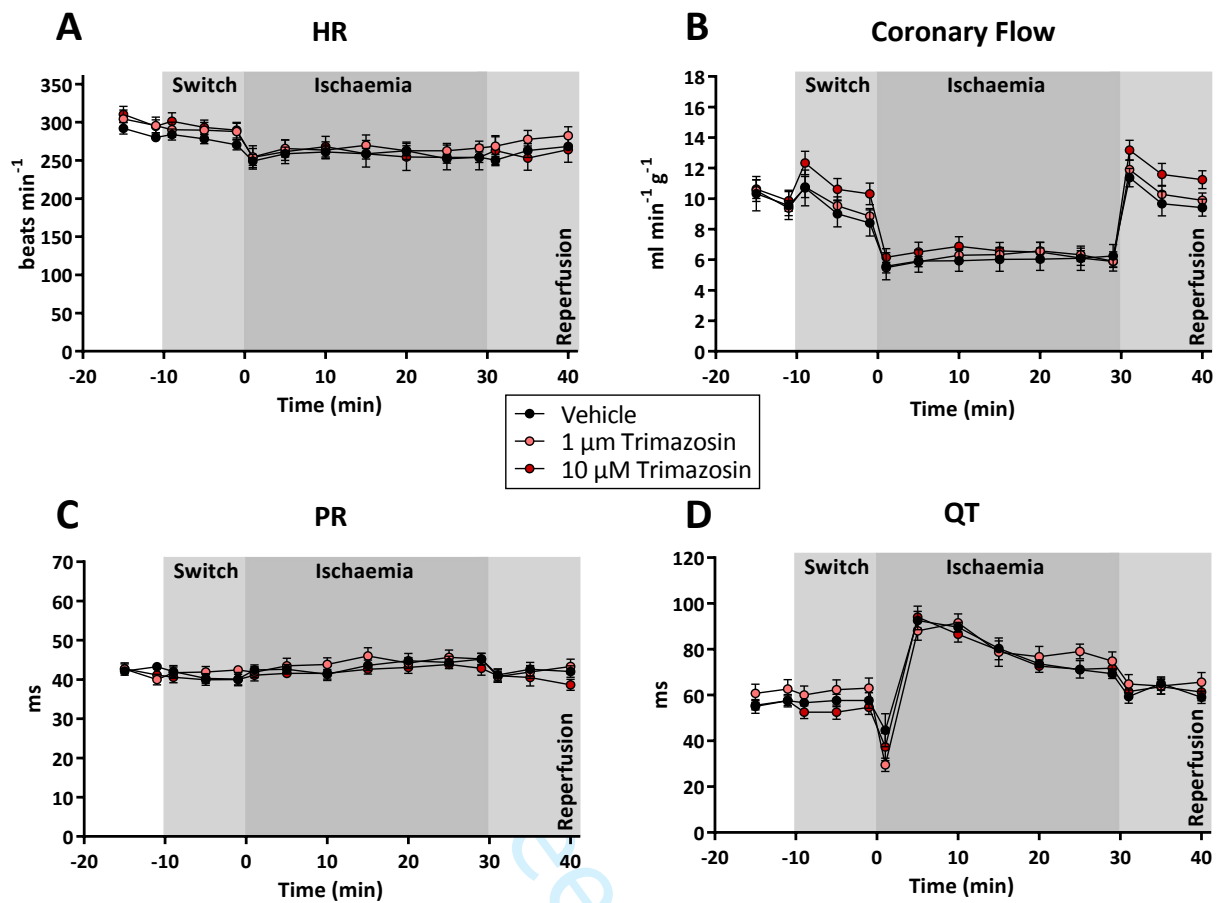
Appendices Figure 1: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μM atenolol. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 12$ per group; mean \pm SEM; * $p < 0.05$ vs. time-matched vehicle. **HR:** heart rate, **PR:** PR interval, **QT:** QT₉₀ interval.

Appendices Figure 2



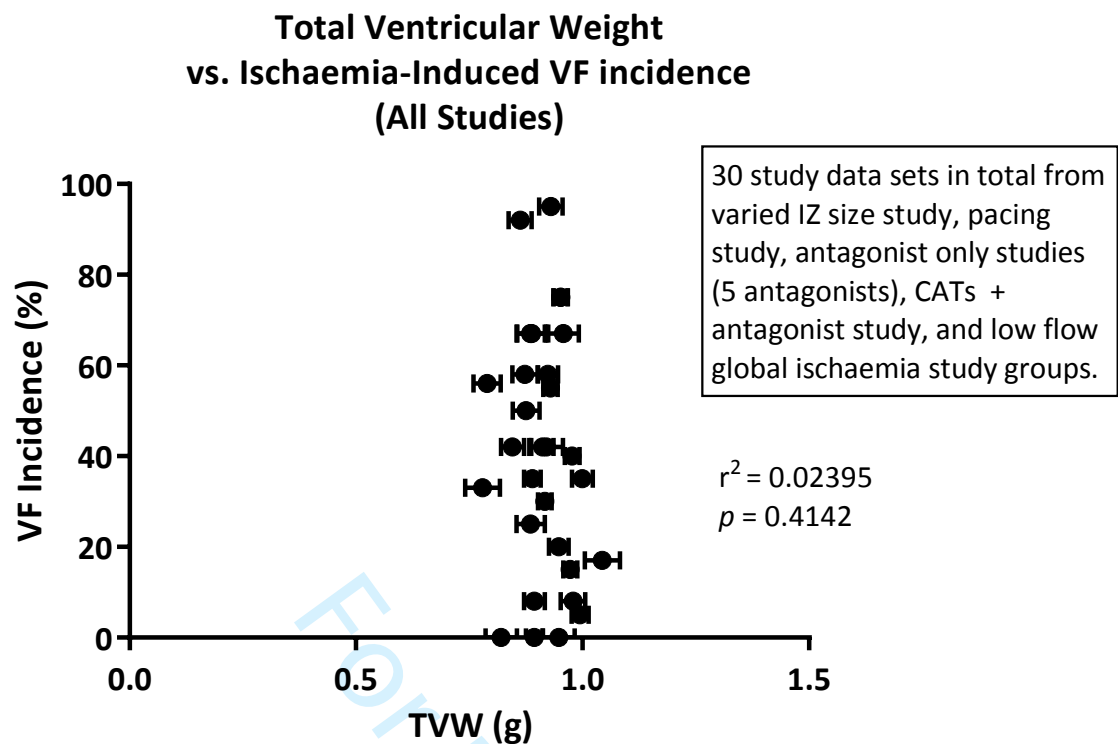
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Appendices Figure 3



Appendices Figure 3: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 µM trimazosin. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 12$ per group; mean \pm SEM. **HR:** heart rate, **PR:** PR interval, **QT:** QT₉₀ interval.

Appendices Figure 4

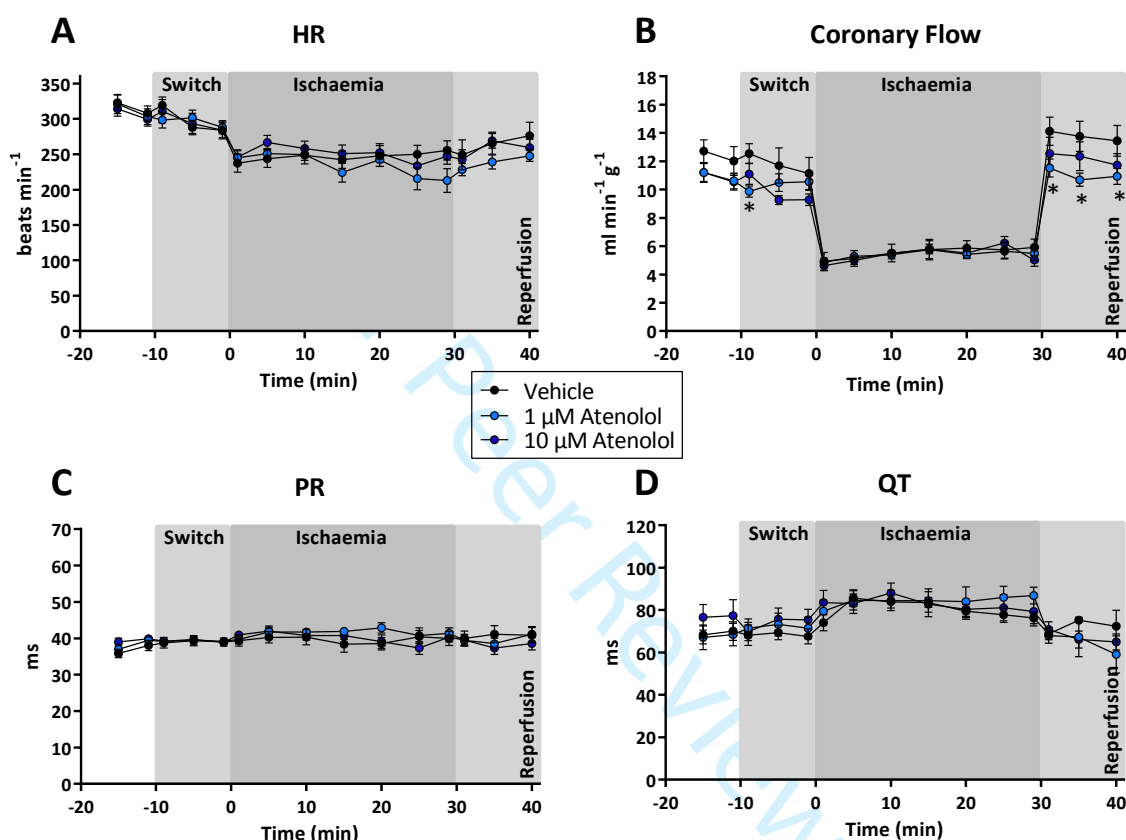


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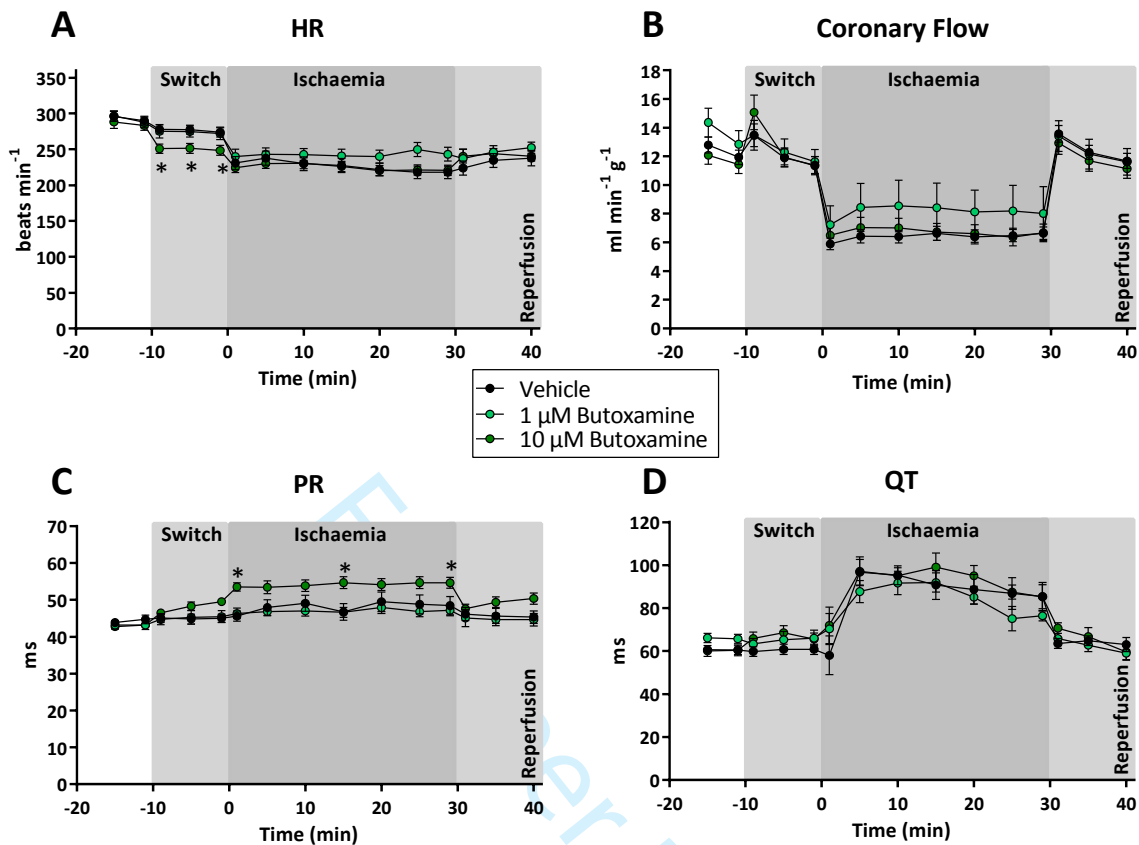
Appendices

Atenolol, butoxamine and trimazosin haemodynamics

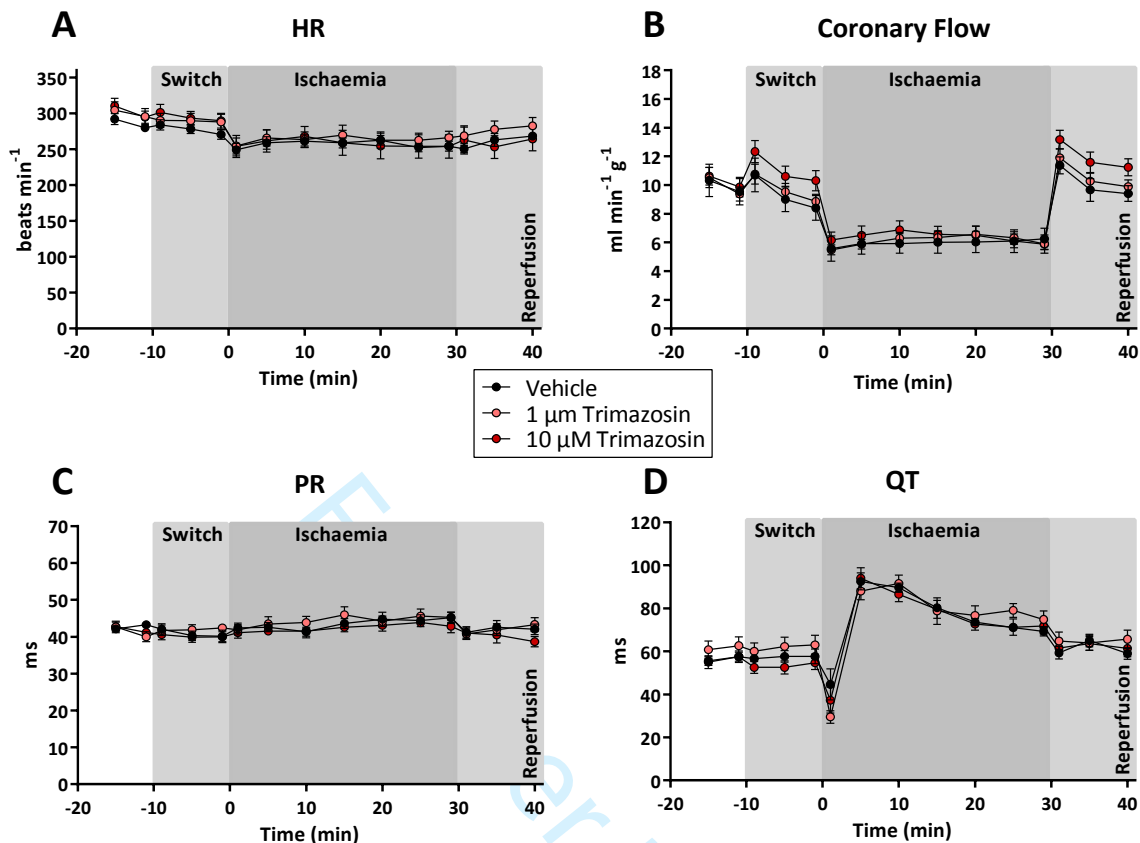
The AR antagonists atenolol (β_1), butoxamine (β_2), and trimazosin (α_1) at 1 μ M had no significant effects on ECG intervals or coronary flow vs. vehicle-perfused hearts (appendices figure 1, appendices figure 2 and appendices figure 3 A-D).



Appendices Figure 1: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μ M atenolol. A) HR, B) coronary flow, C) PR and D) QT intervals. $n = 12$ per group; mean \pm SEM; * $p < 0.05$ vs. time-matched vehicle. **HR:** heart rate, **PR:** PR interval, **QT:** QT₉₀ interval.



Appendices Figure 2: Haemodynamic and ECG changes in hearts with large IZs perfused with 1 or 10 μM butoxamine. A) HR, B) coronary flow, C) PR and D) QT intervals. *n* = 12 per group; mean ± SEM; * *p* < 0.05 vs. time-matched vehicle. **HR:** heart rate, **PR:** PR interval, **QT:** QT₉₀ interval.

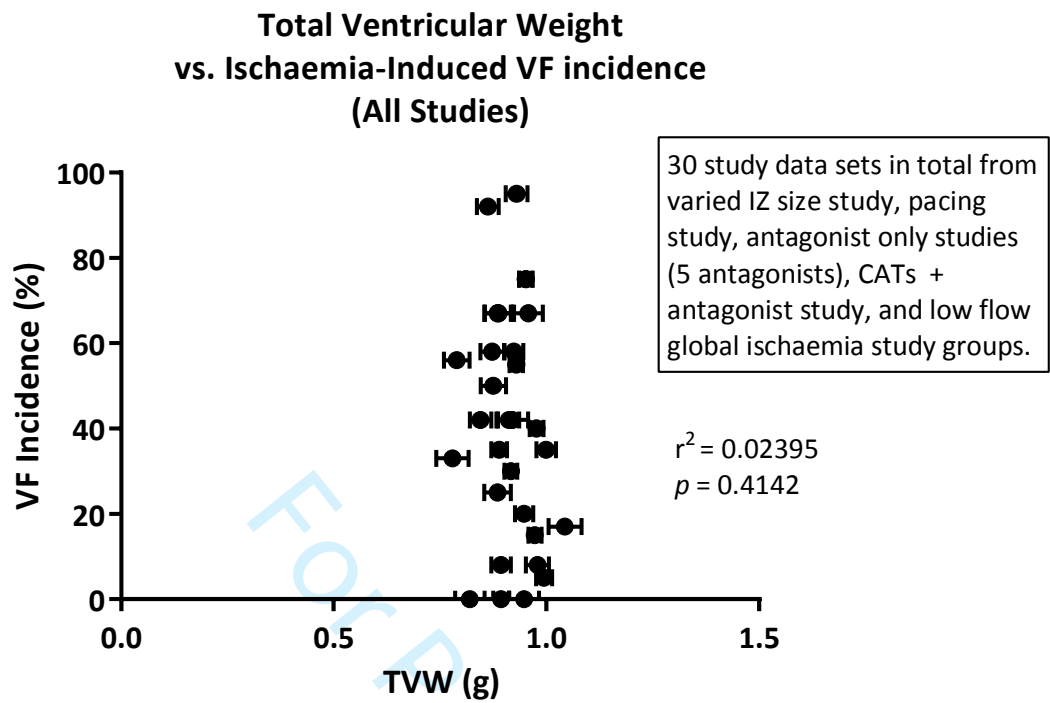


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Relationship between heart weight and VF incidence across all studies

All studies were randomised so any relationship between heart weight and VF incidence will have had no influence on differences in VF incidence between groups, or account for any effects seen. TVW vs. ischaemia-induced VF incidence from hearts from *all* studies used in this paper (30 study data sets) has been plotted (appendices figure 4).

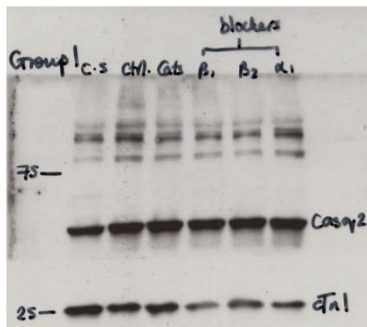
There is no correlation between TVW and ischaemia-induced VF incidence according to Pearson correlation calculation. This shows that TVW is not an independent source of error in our studies. Thus, although body weight and heart size varied in the study overall, this was not a significant source of variance with respect to ischaemia-induced VF incidence.



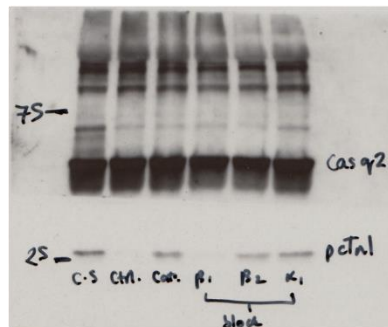
Appendices Figure 4: Relationship between TVW and VF incidence from all hearts used in all studies. **TVW:** total ventricular weight, **VF:** ventricular fibrillation. Mean \pm SEM, Pearson correlation calculation.

Western Blot PDF for review (uncropped blots from **Figure 9**)

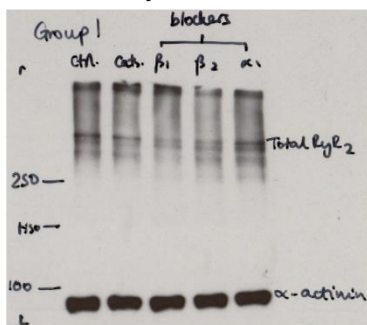
UZ: Total cTnI and Casq2



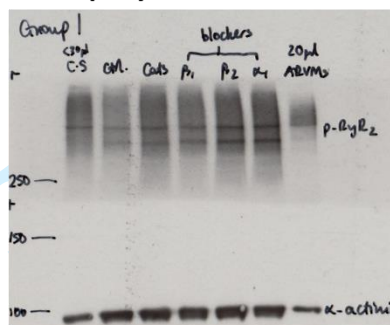
UZ: p-cTnI and Casq2



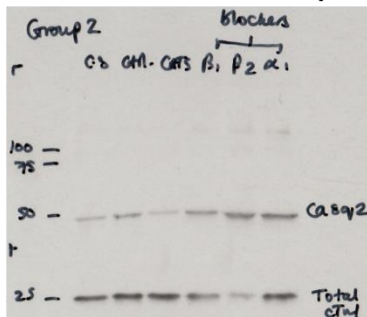
UZ: Total RyR2 and α -actinin



UZ: p-RyR2 and α -actinin



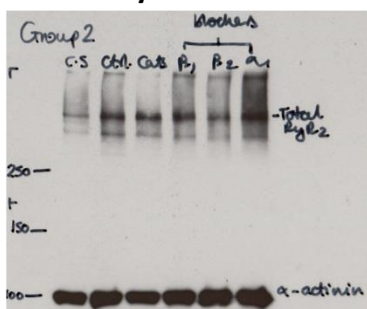
IZ: Total cTnI and Casq2



IZ: p-cTnI and Casq2



IZ: Total RyR2 and α -actinin



IZ: p-RyR2 and α -actinin

